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Genetic Mechanisms for the Maintenance of Behavioral Mating Barriers in Drosophila

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by

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Abstract of the Dissertation

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Professor Yehuda Ben-Shahar, Chairperson

One of the most successful and diverse systems involved in the maintenance of behavioral barriers between closely related animal species is pheromonal communication. In the fruit fly, contact chemosensation input is especially important during sexual decision-making as it allows for the sensing of sex and species-specific non-volatile cuticular hydrocarbons (CHCs), which function as insect pheromones. However, how pheromonal systems support the maintenance of mating barriers is puzzling since any change in either pheromone ligands or their cognate receptors would carry a fitness cost, which should be eliminated by stabilizing selection. To resolve this evolutionary conundrum I hypothesized that pleiotropic genes play a role in both the perception and synthesis of mating-related pheromones. In support of my hypothesis, I found that the gene *Gr8a*, a sexually dimorphic member of the gustatory receptor (*Gr*) family, is expressed in both chemosensory neurons and pheromone-producing oenocytes. Mutations in *Gr8a* lead to courtship related phenotypes that are consistent with a role in sensing inhibitory mating pheromone in males and females and the synthesis of inhibitory pheromones in males. Thus, my findings indicate that a single chemosensory receptor affects not only the perception, but also the production of pheromones in *Drosophila*. My thesis provides a simple solution to an

important unresolved evolutionary question by suggesting that a single major genetic locus can drive the evolution of both pheromones and their receptors and thus the maintenance of behavioral mating barriers between closely related species.

CHAPTER 1: Introduction

The biological species concept, described by Ernst Mayr, and used in the context of this dissertation, defines species as "groups of interbreeding natural populations that are reproductively isolated from other such groups." (Mayr 1942). With over 2,000 species of *Drosophila* in the world and counting, one must wonder how this multitude of *Drosophila* species remain isolated in nature. For some of these species, separation is as simple as being located in different geographical regions of the world. However when the habitat of species overlaps, there may be one of several barriers to species hybridization; these barriers may be pre-zygotic and/or post-zygotic. Defining which of these have contributed to reproductive isolation can be a daunting task, and theoretical and experimental data point to one or both barriers affecting isolation of a population (Nanda and Singh 2012; Palumbi 1994; Panhuis et al. 2001; Michael G Ritchie 2007; Rundle and Nosil 2005; Sobel et al. 2010). While some of the genetic mechanisms that support post-zygotic isolation are well understood, experimental genetic data that explain pre-zygotic behavioral barriers between closely related species are limited.

Pheromones and Species Mating Barriers

One of the most spectacular and diverse systems that evolved to maintain pre-zygotic behavioral barriers across closely related animal species is pheromonal communication. These chemical signatures are used in a wide variety of taxa for many purposes including survival, social organization, kin recognition, alarm signaling, territory marking, mimicry, aggression and sexual behaviors (Chung and Carroll 2015; Symonds and Elgar 2008; Yew and Chung 2015). During sexual communication, pheromones relay important information to potential mates such

as sex, reproductive status, and species (Johansson and Jones 2007). The organism's ability to recognize the quality and variation of these signals is imperative for making correct mating decisions. Chemical signals are identified via families of chemosensory receptors, such as olfactory receptors (*Or*'s), ionotropic receptors *(IR's*), and gustatory receptors (*Gr*'s) in *Drosophila* (Benton et al. 2009; Gao and Chess 1999; K. Scott et al. 2001). If a species is incorrectly identified and a heterospecific mating occurs, offspring may be inviable or infertile, in effect wasting precious energy resources for both of the organisms. Though the importance of pheromonal signal and reception for mate recognition is clear, how these systems have evolved and how they are maintained across closely related species remains an important and fundamental question in animal behavior and speciation genetics.

Pheromones show tremendous diversity between taxa, varying both qualitatively and quantitatively, with the ratio of each of the separate compounds in the overall bouquet differing greatly even in very close species. The evolution of pheromones may proceed in two ways: small gradual changes or large saltational changes (Baker 2002; Symonds and Elgar 2008). In the first case, small changes in pheromonal composition would result in close species having similar pheromones. However, pheromones with functions in species detection must be highly species-specific, and any slight deviations would be eliminated as they would fail to result in successful mate identification. In the second, saltational shifts in pheromones would result in drastically different pheromone composition between closely related species, allowing for proper discrimination of a conspecific or heterospecific organism. However, how these saltational changes in signal would be maintained in a population is unclear.

Theory suggests that in addition to evolutionary changes in signal, selection must also act on pheromone reception as pheromone-receiver pairs must be fine-tuned to each other in order to

support a properly functioning communication system (Boake 1991; Butlin and Ritchie 1989; J. A. Endler 1993). In contrast, a recent study by Niehuis *et al.* in the *Nasonia* wasp suggests one does not need co-evolution of receptor and signal for a functional pair to evolve (Niehuis et al. 2013). Rather, a signal can evolve before a receptor is able to recognize it, yet it can be maintained in the population by female indifference, though the mechanism by which it would achieve fixation remains unclear (Lassance and Löfstedt 2013). Further, one must also consider that pheromone evolution may be driven by the evolution of the receiver (Symonds and Elgar 2008; Tabata et al. 2007). Fixation of a signal may arise as a result of pre-existing sensory bias, in which a novel trait may be maintained due to the sensory system and brain having inherent biases (J. Endler 1998). Regardless of which component of the chemical communication system evolved first or if they co-evolved, in order to understand the maintenance of signal-receiver systems, it is important to gain clarity on the underlying genetics. However, the genetic architecture of these chemical communication systems remains poorly understood for most animal species.

Recent studies have attempted to elucidate the genetic architecture of chemical communication. It is often assumed that independent genes code signal and receiver, and this may seem a compelling hypothesis because often they are located in different tissues. The sensory system that perceives the signal, which is neuronal, is quite different from the system that produces the signal, for example oenocytes in *Drosophila* (Boake 1991). However, due to the high specificity in the mate recognition system, a change in either the signal or receiver will only be beneficial if a new signal is recognizable and relays information to a potential mate, or a new receptor recognizes an old signal. It should also be noted that the signal may change not qualitatively but quantitatively, altering the amount and/or the affinity of a signal to a receiver by

changing a step in the synthesis pathway to a signal (**Figure 1A**). Theory suggests that there will be strong selective pressures against novel variants in a population, as any deviation from either the signal or receptor could cause miscommunication and have negative effects on an organism's ability to obtain a mate, thus presenting a conundrum of how organisms can diversify in the face of stabilizing selection (Brooks et al. 2005). Further, if the pheromonal signal and receiver are controlled by separate genes and evolve via steps, it remains unclear which of these would evolve first and how either would avoid being acted on by stabilizing selection (Symonds and Elgar 2008).

One solution to this problem is genetic coupling, in which linked genes or a pleiotropic mutation underlies signal and receiver variations (**Figure 1B**). Theoretically, this new variant could qualitatively or quantitatively influence both signal and receptor, or the affinity of the signal to the receptor, yet avoid stabilizing selection while maintaining species isolation. Pleiotropy would provide genetic diversity in the signals used by different species as well as a genetic correlation between signal and receiver, which could then be acted on by sexual selection. This would also diminish the effects of hybridization as there would be a lack of recombination between signal and receiver. However, showing this empirically has been difficult and is therefore often rejected in theoretical speciation models. As genomes and genetic tools for model organisms become more readily available, new studies have begun to explore the genetic architecture of signal and receiver/preference and the role of pleiotropy in reproductive isolation and speciation (K. Shaw et al. 2011).

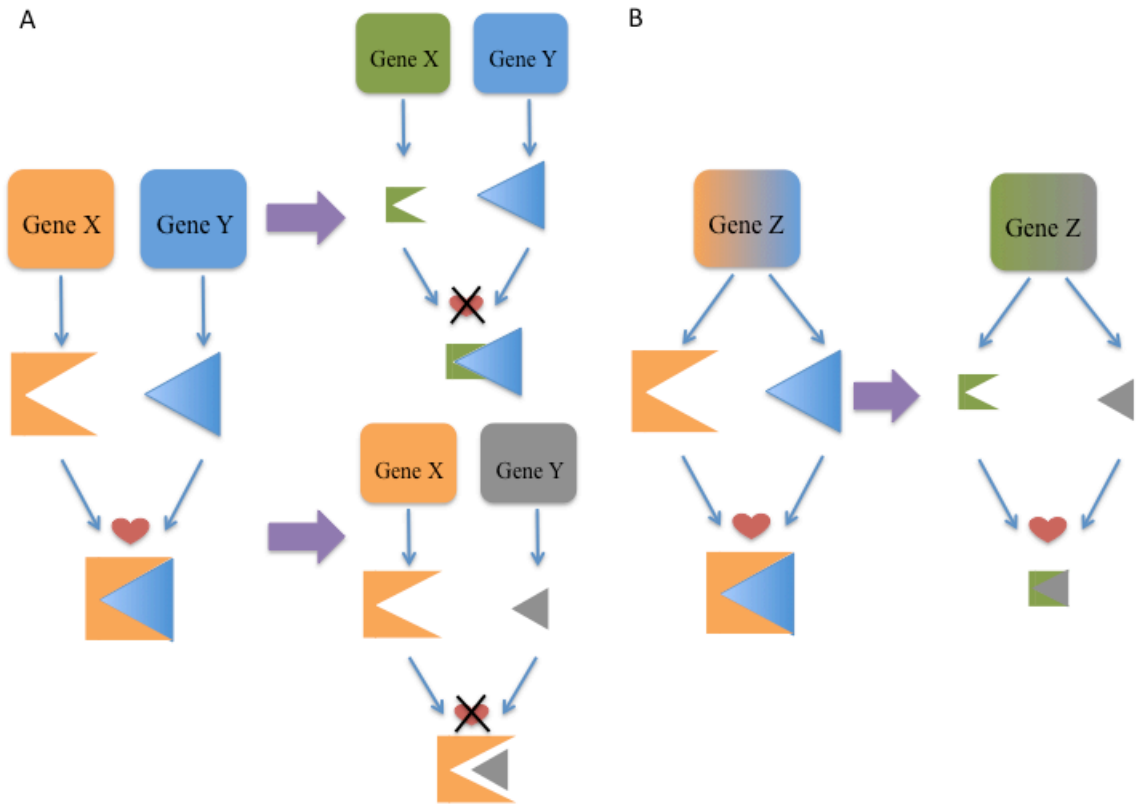

Figure 1. Models for the evolution of signal-receiver systems. (A) Pheromone and receptor are encoded by independent genes. During evolution, if only Gene X or Gene Y evolves (purple arrows), communication between signal and receiver will not be maintained. (B) Alternative pleiotropic model in which Gene Z evolves, affecting both single and receiver yet maintaining communication between them.

Though limited, a handful of empirical studies have provided a strong case for genetic coupling underlying animal chemical communication as measured by mating preference or receptivity. For example, in the Hawaiian cricket *Laupala*, a genetic association was made between QTL for male song and female preference. Wiley *et al.* found physical linkage between four QTL that underlie the pulse rate in male song and the preference for pulse rate by females (Ellison, Wiley, and Shaw 2011; Wiley, Ellison, and Shaw 2012). Though this study did not imply that a single major gene affects both signal and preference, the genetic coupling of groups of genes may facilitate the maintenance of behavioral barriers once two or more species have evolved.

An additional study supporting genetic correlation between preference and signal is found in Lepidoptera. For example, *Heliconius* males make courtship decisions based on wing color pattern, and varying color patterns often result in assortative mating between species. Kronforst *et al*. (2006) found that wing color pattern and the male preference for wing color pattern are affected by variation in the developmental gene, *wingless*. However, they suggested that it is unlikely that a morphological trait and sensory process would be strictly pleiotropic. Regardless, evidence for a tight genetic link between preference and trait remains exciting in understanding how phenotypic diversity can persist between species despite stabilizing selection pressures and hybridization (Jiggins et al. 2001; Kronforst et al. 2006; Naisbit, Jiggins, and Mallet 2003). In addition, a study in *Heliothis* moths by Gould *et al.* (2010) reported sexual isolation between two species of moths due to four odorant receptor genes under tight linkage on a single QTL. As moths with divergent pheromonal signals are selected against, it was puzzling how there could be such a great diversity in nature. This study reported that the change in odorant receptor genes resulted a change in pheromonal response to Z9-16:Ald, Z9-14:Ald, and Z11-16:OAc and ultimately resulted in isolation between the two species (Gould et al. 2010). Thus far, these experiments have supported the physical correlation between genes that affect signal and/or receiver, but have not provided evidence for the existence of a single gene affecting both aspects of the communication system.

Though studies supporting pleiotropy as the genetic architecture underlying communication systems are limited, there are two recent studies supporting this hypothesis. In Japanese madaka, *Oryzias latipes*, an 11-bp deletion in the *somatolactin alpha* (*Sla*) gene causes a change in pigmentation, and also a change in mate preference. Mutants for *Sla* assortatively mate, showing that one gene can affect both trait and preference, contributing to reproductive

isolation (Fukamachi et al. 2009). Further, in *Drosophila*, *desaturase1 (desat1)*, a gene encoding a desaturase that controls the synthesis of monoenes, including the important male CHC 7-tricosene, was shown to also have roles in flies ability to discriminate between males and females. Eliminating the expression of *desat1* lowered the amount of pheromones detectable on the fly cuticle and also eliminated reproductive barriers between select species (Billeter et al. 2009; Bousquet et al. 2012; Marcillac, Grosjean, and Ferveur 2005). Not only does *desat1* play a major role in pheromone production, but is also important for discrimination between possible mates, thus illuminating its role in both signal and perception and reproductive isolation.

Often, tissues involved in signal production and perceptions are very different, and therefore pleiotropy may seem unlikely. For example, in *Drosophila* pheromones are produced by oenocyte cells under the culticle in the abdomen and are detected by chemosensory receptors, including olfactory receptors (*Or's*), ionotropic receptors (*Ir's*), and gustatory receptors (*Gr's*). While *Or's* and *Ir's* are mostly confined to the head region, *Gr's* are distributed in gustatory sensilla on the proboscis, wing margins, legs, and female ovipositor (**Figure 2**). Therefore, it may seem unlikely that the same gene or genetically coupled genes could affect both pheromone and receiver. However, recent studies, including ours, suggest atypical expression of *Gr's* in *Drosophila*. *Gr's* were originally named so because the first receptors that were characterized were found exclusively in taste neurons. However, there is now a growing body of evidence for their non-canonical expression and function (Jones et al. 2007; Miyamoto and Amrein 2014; Montell 2013; Ni et al. 2013; Thorne and Amrein 2008). What are they doing in locations other than in peripheral neurons? Interestingly, our research opens up an exciting avenue of research suggesting some chemoreceptor genes may actually be involved in pheromone production. As pheromones are very important for mating in many species, this may also have implications for

reproductive isolation between species, which is a topic I explore in my thesis.

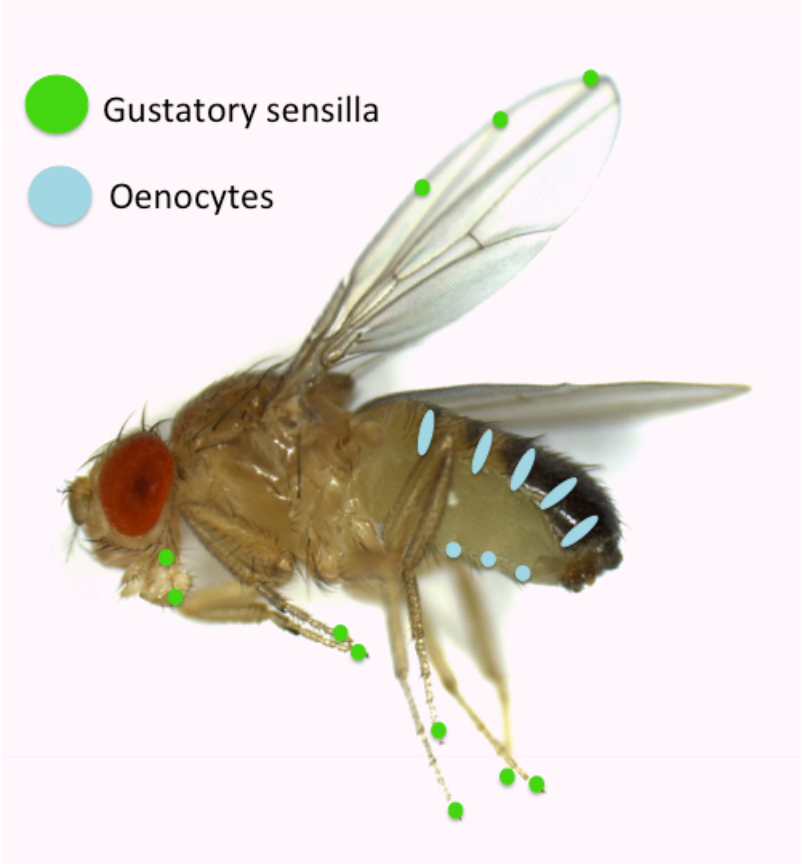

Figure 2. Contact chemosensory ("gustatory") sensilla and oenocytes. *Drosophila* gustatory sensilla are located on the proboscis, labellum, tarsal segments of the legs, and wing margins of males and females. They are also located on the ovipositor in the female (not shown). Oenocytes are located under the cuticle in the abdomen in both sexes. Green, gustatory sensilla and blue, oenocytes.

Based on the atypical expression of gustatory receptors in the abdomen, and the known location of pheromone-producing oenocytes also in the abdomen, this thesis empirically tests the hypothesis that a single chemosensory receptor gene can impact both signal (pheromone) production and perception. As communication systems are very important in species isolation, testing is then completed on a chemosensory gene's role in behavioral pre-zygotic isolation in *Drosophila* species.

The Signal: Cuticular Hydrocarbons

When *Drosophila* encounter one another in nature they must have the ability to quickly determine several pieces of information before deciding if courtship and mating is warranted. For if they attempt copulation with the wrong sex, the wrong species, or even a female which has previously mated, mating attempts will only waste energy and precious resources. *Drosophila melanogaster* utilize multiple sensory modalities to learn important information about potential mates, including visual, auditory, chemosensory, and mechanosensory information (Gailey, Lacaillade, and Hall 1986; Krstic, Boll, and Noll 2009). The different signals vary between species, however mating interactions are often mediated by pheromones and chemosensory receptors. When chemosensory systems are involved in mating and reproduction they may be functioning as a barrier to genetic exchange (Smadja and Butlin 2009).

In *Drosophila*, courtship is a key pre-zygotic species barrier in which chemical communication is important for the correct identification of potential mates. It is a genetically-driven, innate behavior in which males follow a specific sequence of events during courtship of females, differing by species. The female will either accept the male's offer allowing him to copulate with her or will reject him by kicking him, flying away, or extruding her ovipositor (Greenspan and Ferveur 2003; Lasbleiz, Ferveur, and Everaerts 2006; Spieth 1974). During courtship, *Drosophila* use pheromones, cuticular hydrocarbons (CHCs) of various chain lengths, to identify potential mates.

CHC's are made in the oenocyte cells (**Figure 1**), which are large secretory cells found under the cuticle of the abdomen in close association with fat body cells. CHC's are produced via a series of enzymatic reactions (Martins and ramalho-Ortigao 2012; Tillman et al. 1999). They form a waxy layer on the cuticle and though they help to prevent desiccation by controlling

water loss (Chung and Carroll 2015), they also function in social behaviors including mating, aggression, and aggregation as they vary according to age, sex, diet, geographic origin, and species (Dahanukar and Ray 2014; J.-F. Ferveur 2005; Jallon and David 1987).

Some of the best studied non-volatile pheromones with roles in mating behaviors in *Drosophila melanogaster* include 7,11-dienes in females and 7-tricosene in males. 7-11-heptacosadiene (7,11-HD) and 7,11-nonacosadiene (7,11-ND) serve as aphrodisiacs for conspecific males but are inhibitory for heterospecific males (C Antony et al. 1985; Marcillac and Ferveur 2004; Marcillac, Houot, and Ferveur 2005). In *D.melanogaster* males, the monoalkene (Z)-7-tricosine (7-T) is the most abundant and serves as an aphrodisiac for females but an anti-aphrodisiac for males during courtship (J.-F. Ferveur 2005; Grillet, Dartevelle, and Ferveur 2006; Jallon and David 1987). The specific chemosensory receptors responsible for detecting 7-T are speculated to be *Gr32a* and *Gr33a*, though it is likely that there are others that remain unknown (Lacaille et al. 2007; Lacaille, Everaerts, and Ferveur 2009). Though much investigation has been completed on these few compounds, the majority of compounds in the *Drosophila* CHC profile have unknown functions (C Antony et al. 1985; Claude Antony and Jallon 1982; Everaerts et al. 2010).

Several compounds of recent interest include methyl-branched cuticular hydrocarbons (mbCHC's). Chung *et al.* (2014) found that these mbCHC's play a role in both desiccation resistance and mate choice in *Drosophila serrata*. When males were perfumed with 2MeC26, they had increased mating success. Interestingly, they also suggest a role for mbCHC production in maintaining reproductive isolation between *Drosophila serrata* and *Drosophila birchii*, but suggest that mbCHC's are not sufficient for the mating barrier and it is likely that other factors involved in courtship and mating play a role as well (Chung et al. 2014). The select non-volatile

contact pheromones mentioned above are detected by *Drosophila* pre-mating and have implications for behavioral pre-zygotic isolation; however, there are pheromones which are transferred from the male to the female upon mating which influence behavioral isolation as well.

During mating male *Drosophila* transfer ejaculate to the female that is composed of sperm cells, sex peptides and proteins, as well as volatile and non-volatile compounds (Poiani 2006; Wolfner 2002, 2007). Once ejaculate is transferred, it has reproductive, physiological or behavioral effects on the female, and often the compounds can be detected on the female cuticle for several hours or in some cases days, preventing other males from attempting copulation and thus ensuring paternity for the mated male. Two of these lipids have shown to serve as anti-aphrodisiacs, inhibiting other males from courting and/or attempting copulation with a previously mated female. The first, cis-vaccenyl acetate (cVA), is perceived by the olfactory system and is involved in multiple social interactions including stimulating female receptivity, hindering male courtship, facilitating aggression, and inducing aggregation (Bartelt, Schaner, and Jackson 1985; Butterworth 1969; Ejima 2015; Jallon, Antony, and Benemar 1981; Kurtovic, Widmer, and Dickson 2007; Wang and Anderson 2010). Levels of cVA are only detected in the female reproductive tract for several hours after mating (Vander Meer et al. 1986); therefore it is likely that several other transferred compounds are at play in the inhibition of mating attempts from subsequent males.

Recently, CH503 (3-*O*-acetyl-1,3-dihydroxy-octacosa-11,19-diene) has been shown to also be transferred to the female during mating and inhibit subsequent mating attempts by other males. This compound is not detectable on virgin females, but is the longest-lasting detectable change on the mated female's cuticle (Yew et al. 2009; Yew and Chung 2015). These long lasting effects make CH503 an important player in the maintenance of a mated female's

inhibitory status. Though there exists evidence for a post-mating role of both CVA and CH503, there remains no consensus on their roles pre-mating.

CHC's allow for chemical recognition of conspecifics during courtship as the pheromonal composition of different *Drosophila* species varies quantitatively and/or qualitatively, inhibiting or stimulating the opposite sex or species. Among species, CHC's vary in hydrocarbon chains, either in the position and number of double bonds or the length of the carbon chain, and in some *Drosophila* species are sexually dimorphic. Interestingly, males of species whose pheromones are sexually dimorphic will not court females whose pheromones are sexually monomorphic, and vice versa, facilitating behavioral reproductive isolation (Cobb and Jallon 1990; Coyne, Crittenden, and Mah 1994; Dahanukar and Ray 2014). When the oenocytes are ablated pheromones are not produced, and behavioral isolation between species is lost, suggesting the importance of cuticular hydrocarbons for species recognition and divergence (Billeter et al. 2009; Etges and Jackson 2001).

The Receiver: Chemosensory Receptors

Insect chemosensory receptors consist of multigene familes of olfactory and gustatory receptors. These *Gr's* have seven transmembrane spanning domains, but in flies have an inverted orientation and are not homologous to G-protein coupled receptors (GPCR's) (Benton et al. 2006; Vosshall et al. 1999). Current understanding suggests that these receptors act as ligand-gated ion channels, rather than GPCR's (Benton 2008; Sato et al. 2008). *Drosophila melanogaster* has 60 *Or* genes and 60 *Gr* genes, coding for 62 and 68 proteins, respectively. (P. J. Clyne 2000; Dunipace et al. 2001; Gao and Chess 1999; Robertson, Warr, and Carlson 2003; K. Scott et al. 2001; Vosshall et al. 1999). Both families are very divergent in amino acid sequence

(Robertson, Warr, and Carlson 2003) and are housed in sensilla in various chemosensory regions of the fly. Recent studies have suggested that they are also distributed in non-chemosensory parts of the fly including neurons in the brain, abdominal multidendritic neurons, neurons associated with the Johnston's organ, and antennal neurons playing non-gustatory roles (Dunipace et al. 2001; Fujii et al. 2015; Jones et al. 2007; Miyamoto et al. 2012; Miyamoto and Amrein 2014; Park and Kwon 2011a, 2011b; Shimono et al. 2009; Stocker 1994; Thorne and Amrein 2008).

A handful of *Gr's* have been found to have functions in detecting sweet and bitter taste as well as pheromones (Bray and Amrein 2003; Dahanukar et al. 2007; Isono and Morita 2010; Miyamoto and Amrein 2008; Moon et al. 2006; Slone, Daniels, and Amrein 2007), but over half of the *Gr* repertoire still have unknown function. Further, many of the ligands of *Gr's* have not yet been classified, though they can be divided into three groups: sugars, bitter compounds, and pheromones. Those *Gr's* involved in pheromone detection are likely to participate in mating behaviors as pheromones are used by individuals to detect potential mates. Though several *Gr's* are speculated to sense pheromones, including *Gr68a*, *Gr32a*, and *Gr33a*, and their mutants have shown to be deficient in courtship behaviors, there remains no confirmation of the specific ligands for these receptors (Bray and Amrein 2003; Miyamoto and Amrein 2008; Moon et al. 2009).

Gustatory receptor neurons (GRN's) consist of a cell body with a single dendrite extending to the tip of gustatory sensilla where a single pore is located. The different types of sensilla are named based on their length: long (l-type), intermediate (i-type), and short (s-type) and contain two-four GRN's, a mechanosensory neuron, and accessory cells (Montell 2009). Current understanding holds that rather than working independently, multiple *Gr's* together form a functional receptor unit (Jiao et al. 2008; Lee, Moon, and Montell 2009; Poudel et al. 2015;

Shim et al. 2015). Shim *et al.* concluded that the expression of three Gr's-*Gr8a*, *Gr66a*, and *Gr98b*, are all required for the appropriate response to l-cavanavine, a plant-derived lethal analog of L-arganine that is mistakenly incorporated into the genome (Shim et al. 2015). In addition, a study by Poudel *et al.* suggest that *Gr33a*, *Gr66a*, and *Gr93a* together generate a receptor for umbelliferone, a phenylpropanoid found in some plants that is avoided by *Drosophila* (Poudel et al. 2015). It is unclear whether or not these complexes require one or two broadly-tuned *Gr's* as in the two subunit *Or* complex, which requires one broadly-tuned receptor, *Or83b* (Benton et al. 2006). In Shim's model there is one broadly required *Gr*, *Gr66a*, while the other two *Gr's* are more narrowly tuned. Yet in Poudel's model, two of the *Gr's* are broadly tuned while the other is more narrowly tuned to a specific ligand. Though new studies support the existence of core *Gr's*, the number of *Gr* subunits required and the extent to which functional *Gr* complexes form to respond to aversive compounds remains unclear (French et al. 2015; Moon et al. 2009; Weiss et al. 2011).

Pleiotropy in Drosophila Chemical Communication

My thesis work is focused on *Drosophila melanogaster* and related species as a model to understand the genetic bases for the coupling of pheromone perception and production, and suggests the existence of pleiotropy underlying sexual communication systems in this phylogenetic group. Here I ask if specific chemosensory receptors can contribute to the perception and production of sexual signals. The presented studies demonstrate a role for a specific gustatory receptor gene in regulating female mating choices, via contributions to both the production and perception of mating-related chemical signals. To my knowledge, data

presented here represents the first experimental evidence for the possible contribution of pleiotropic chemoreceptors to behavioral mating barriers between closely related species.

How chemoreceptors might contribute to pheromone production is not yet known. One possible cellular mechanism could be via positive or negative feedbacks in pheromone-producing tissues via cell-intrinsic processes associated with regulation of specific synthetic steps or the secretion/transport of specific CHCs. Rather than the evolution of gustatory receptor genes directly impacting the synthesis of specific pheromone components, it would indirectly affect the overall pheromonal profile of individuals by possibly shifting the relative amounts of specific CHCs due to changes in ligand affinities, or the molecular interactions with downstream signaling molecules. Future biochemical and cellular studies should aim to provide further insight into the mechanism by which specific chemosensory genes could act as pleiotropic factors, especially in oenocytes.

Questions addressed in my thesis are important to understand how the diversity of life is maintained. As species evolve, both pheromones and their receptors have to co-evolve to support robust species-specific mating. Therefore, because any changes in either the receptor or the pheromone could result in decreased fitness, the likelihood that even small changes in pheromonal blends could lead to diversification of mating pheromones seems unlikely. New studies highlighting pleiotropy as the underlying genetic architecture of species and species maintenance is an excellent alternative to independent genes as stabilizing selection would not eliminate diversity in signal/receiver systems. However, pleiotropy underlying the maintenance of species has been difficult to study empirically as it is often difficult to distinguish between pleiotropy and tight genetic correlations. This study adds a significant contribution to the very small body of literature that has recently investigated and found solid evidence for pleiotropy in

chemical communication. Advances in genomic tools in model organisms will make way for exciting advances and shed light on this particular area of study.

CHAPTER 2: The coupling of pheromone production and perception via chemoreceptor pleiotropy

Introduction

Pheromonal chemical communication is essential for sexual reproduction and fitness in diverse animal species because it is effective in communicating important information such as sex, mating status, and species identity. In insects, the perception and synthesis of pheromones are mediated by independent tissues via different molecular pathways. Consequently, it is typically assumed that pheromones (the "signal") and pheromone receptors (receiver) are encoded by different genes, which may or may not be genetically linked (Blows 1999; Butlin and Ritchie 1989; Kronforst et al. 2006; Lande 1981; Lofstedt et al. 1989; M G Ritchie 2000; Ryan 1988; K. L. Shaw and Lesnick 2009; Sureau and Ferveur 1999).

To date, the majority of published evolutionary and ecological theoretical models are primarily focused on the initial formation of ligand-receptor coupling under specific ecological contexts. However, once the coupling of specific ligands and their cognate receptor are established, they should retain the capacity to evolve at the population level (Kirschner, Gerhart, and Gerhart 1998; Wagner and Altenberg 1996). Therefore, it is often assumed that communication systems that are based on the specific coupling of signals and receptors should retain the capacity to respond to selective pressures via adaptive phenotypic changes or via neutral genetic drift. A recent study by Niehuis *et al*. suggests that pheromone diversity can theoretically evolve and be maintained in a population without being selected against, and without changes in the receiver (Niehuis et al. 2013). Although this study provided support for how new chemical signals might evolve in a population, it did not provide any explanations into how specific signals such as mating pheromones could be fixed in a population, or how natural

and/or sexual selections might have lead to coupled adaptive changes in the receptor and the associated mating preference (Lassance and Löfstedt 2013).

Consequently, how robust species-specific pheromonal communication systems that are essential for identifying potential mates can evolve as populations diversify remains unknown. This is especially puzzling since theoretical models stipulate that pheromonal systems should resist molecular evolution since even small changes in either the ligand or the receptors would confer a fitness cost, and thus should be eliminated from the population by stabilizing selection (Brooks et al. 2005). Therefore, how distinct pheromone-receptor systems have evolved to support mating barriers across closely related species is puzzling (Johansson and Jones 2007; Niehuis et al. 2013; Symonds and Elgar 2008). One possible mechanistic solution to this evolutionary conundrum is that the perception and production of pheromones are genetically linked via pleiotropy (Alexander 1962; Boake 1991; Fukamachi et al. 2009; Marcillac, Grosjean, and Ferveur 2005; K. Shaw et al. 2011). If true, pleiotropy could maintain the robust coupling of pheromones and their cognate receptors as the population diversifies.

Fruit flies are an ideal model organism to test this hypothesis as they have simple genetics, short generation times, pheromonal differences between species and sexes, and robust innate courtship behaviors. In *Drosophila*, cuticular hydrocarbons (CHC's) are the primary pheromones that communicate mating related information such as gender or species identity. CHC's are synthesized in specialized cells termed 'oenocytes', which are located mostly in the adult abdomen (Wicker-Thomas et al. 2015). Chemical analyses revealed a complex repertoire of CHC's on the cuticle of *D.melanogaster*. Though one of the primary roles of CHC's is desiccation resistance (Gibbs 1998, 2002), some CHC's have been shown to function in courtship behaviors, including the female aphrodisiac pheromone 7-11-heptacosadiene (7,11-

HD) and the male inhibitory pheromone *Z*-7-tricosene (7-T) (Billeter et al. 2009; J.-F. Ferveur 2005; Kent et al. 2008; Krupp et al. 2008). However, the molecular receptors for both pheromones remain unknown. In addition, two CHCs, CH503 and 11-cis-vaccenyl acetate (cVA), are transferred from males to females during mating and act as inhibitory pheromones (Billeter et al. 2009; Kurtovic, Widmer, and Dickson 2007; Zawistowski and Richmond 1986). While the volatile cVA is detected by the olfactory system, the molecular receptor for the non-volatile CH503 is detected by *GR68A,* (Shankar et al. 2015; Yew et al. 2009) and likely other gustatory receptors. When oenocytes are ablated, pheromones are not produced and species isolation is lost (Billeter et al. 2009).

Drosophila melanogaster has three multigene families encoding chemosensory receptors: 62 Olfactory receptors (*Ors*), 68 Gustatory receptors (*Grs*), and 61 Ionotropic receptors (*Irs*) (Benton et al. 2009; P. J. Clyne 2000; Dunipace et al. 2001; Gao and Chess 1999; Robertson, Warr, and Carlson 2003; K. Scott et al. 2001). A handful of *Grs* have been characterized as receptors for sweet and bitter compounds as well as pheromones, but the sensory function for most *Grs* remains unknown (Billeter et al. 2009; Dahanukar et al. 2007; Lee et al. 2012; Miyamoto and Amrein 2008; Montell 2009; Moon et al. 2006; Watanabe et al. 2011). The majority of *Gr* genes are expressed in contact chemosensory neurons in the proboscis, wing margins, legs, and female ovipositor. Surprisingly, recent studies have suggested that some *Grs* are also expressed in non-chemosensory cells such as neurons in the brain, abdominal multidendritic neurons, and neurons associated with the Johnston's organ (Dunipace et al. 2001; Jones et al. 2007; Montell 2009; Park and Kwon 2011b; Thorne and Amrein 2008; Vosshall and Stocker 2007). The 'atypical' expression of *Grs* outside the canonical chemosensory system suggests that these genes have extra-chemosensory functions. Since some GR's have been

characterized as pheromone receptors, some pleiotropic pheromone-sensing *Gr's* may also play a role in the regulation of production of specific CHC's.

Results

1.1 *Gr8a is a sexually dimorphic gene enriched in the male abdomen*

To identify receptors that might contribute to pheromone synthesis we first performed an RT-PCR screen to identify *Gr* genes expressed in the abdomens of male and female *Drosophila melanogaster*. Thirty-two *Gr's* showed positive expression in the abdomen, and we identified 16 *Gr* genes that showed a clear sexually dimorphic expression pattern (**Table 1**). I focused on these receptors first as sexually dimorphic traits are likely to play a role in sexual selection. One of the identified genes, *Gr8a*, was recently implicated in the sensory detection of the plant-derived insect feeding deterrent L-canavanine (Lee et al. 2012; Shim et al. 2015). Although the natural ligand of *Gr8a* is not known, studies indicated that it is located in a chromosomal segment on the X chromosome that is implicated in speciation in some *Drosophila* subgroups, and seems to be under natural selection in wild *D. melanogaster* populations (Jeong et al. 2008; Takahashi et al. 2009). Thus, I focused on *Gr8a* as the first candidate gene to test my hypothesis that some *Grs* have pleiotropic functions in the perception and synthesis of specific CHCs.

To identify the specific cells that express *Gr8a,* I used the UAS-GAL4 binary expression system (Brand and Perrimon 1993) to drive the expression of a reporter gene (GFP) by the previously published *Gr8a*-GAL4 line (Weiss et al. 2011). Confocal imaging analysis revealed that *Gr8a* promoter is active in two "gustatory"-like paired neurons, that project their sensory cilia into single contact chemosensory bristles in the pre-tarsal segment of the foreleg, as well as several "gustatory"-like chemosensory neurons in the proboscis (mouth parts) of males and females. *Gr8a* expression is also enriched in oenocyte-like cells in the male abdomen, but not in

the female (**Figure 1**). The co-localization of *Gr8a* with the oenocyte marker *desaturase1* (*desat1*) (Billeter et al. 2009) in the male abdomen confirmed that *Gr8a* is expressed in pheromone-producing cells in addition to its expression in the canonical chemosensory neurons (**Figure 2**). Together, these data indicate that *Gr8a* plays a pheromone-sensory role in both males and females, and possibly pheromone synthesis only in males.

Table 1. Candidate *Gr* genes in virgin male and female abdomen. Plus sign indicates presence of PCR product and minus signs indicates PCR product not detected.

	Mated Male	Virgin Male	Mated Female	Virgin Female
GR2a	-	-	+	+
GR8a	+	+	-	-
GR10a	+	+	+	+
GR10b	+	-	-	-
GR21a	-	-	-	+
GR22a	-	+	-	-
GR22e	+	+	+	+
GR22f	-	-	+	-
GR23a	-	-	+	-
GR28a	-	-	+	-
GR28b	-	-	+	-
GR32a	+	-	-	-
GR36c	+	+	-	-
GR58c	+	+	+	+
GR59a	+	+	+	+
GR59b	+	+	+	+
GR59f	+	-	-	-
GR61a	+	-	+	-
GR63a	+	+	-	-
GR64a	+	+	-	-
GR64b	+	+	-	+
GR64c	+	+	+	+
GR64d	+	+	-	-
GR66a	+	+	+	+
GR89a	+	+	+	+
GR93a	+	-	+	+
GR93d	+	+	+	+
GR97a	+	+	+	+
GR98a	+	+	+	+
GR98b	-	-	+	+
GR98c	+	+	+	+
GR98d	+	+	+	+

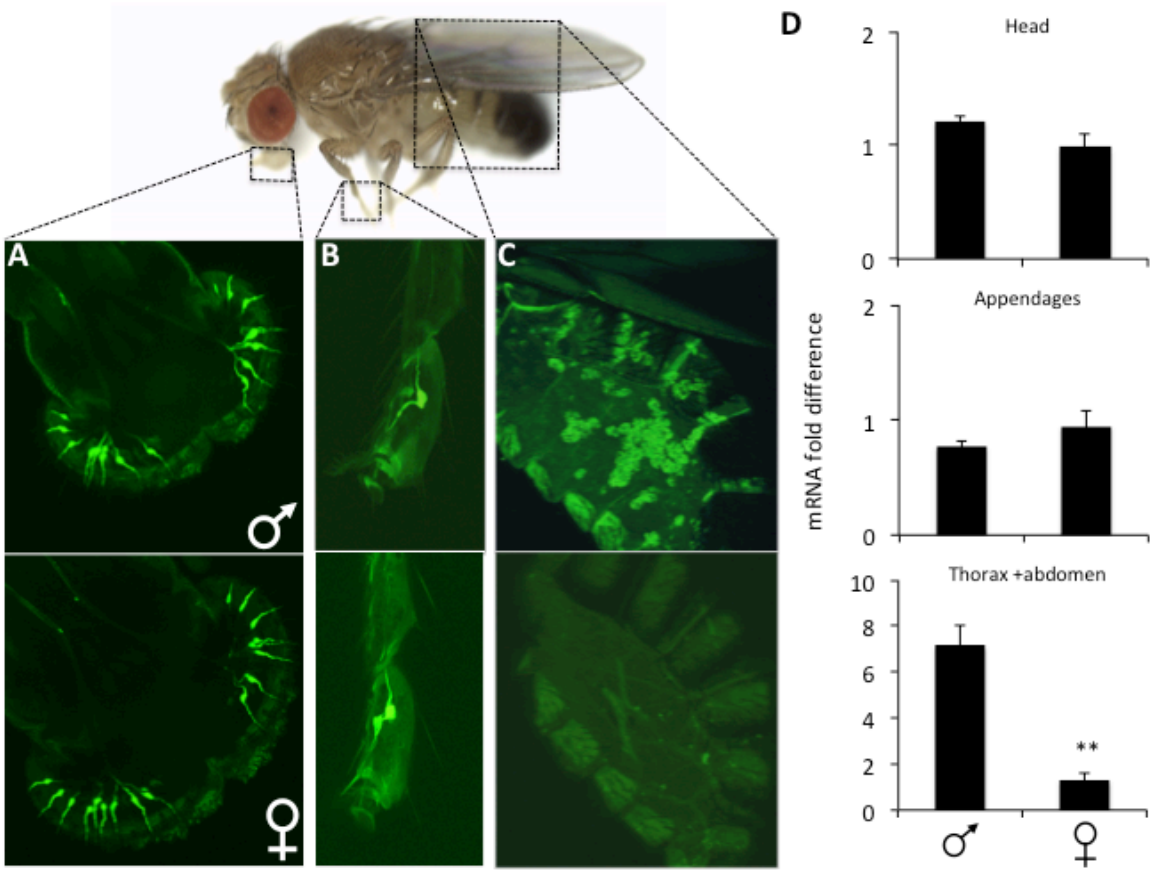

Figure 1. *Gr8a* is sexually dimorphic in the abdomen of *D.melanogaster*. (A,B,C) *Gr8a* expression in proboscis (A), foreleg (B), and abdomen (C) in males (top) and females (bottom). Genotype imaged: *+;Gr8a-GAL4>UAS-YC2.1;+*. (D) Fold change in expression between sexes by body part. *, p<0.05 Mann Whitney Rank Sum Test.

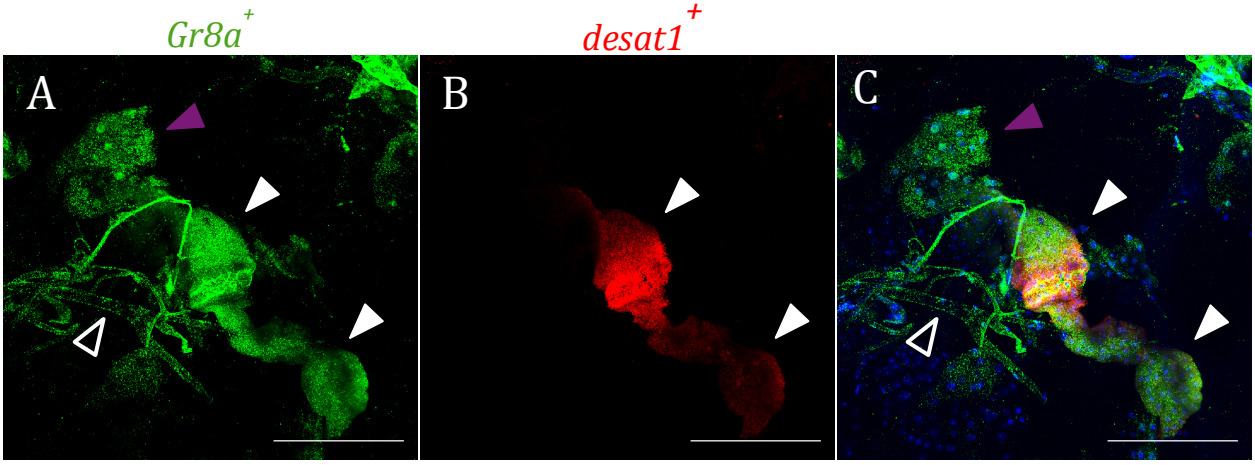

Figure 2. *Gr8a* is co-expressed with *desaturase1*. (A) *Gr8a>GFP* (B) *desat1>luciferase* (C) Co-expression (blue, nuclear DAPI stain). White arrowhead, oenocytes; Purple arrow, fat body; Open arrowhead, trachea. Genotype imaged: *Gr8a-GAL4/GFP;luciferasedesat1/GFP*. Scale bar = 100 um.

1.2 ***Gr8a and the cells that express it are required for normal mating behaviors***

1.2a Gr8a is required for normal female sexual behaviors

I next asked whether *Gr8a* and the neurons that express it are required for normal female mating behavior. To address this, I examined the effects of blocking neuronal transmission in *Gr8a*-expressing neurons in females with tetanus toxin (TNT) (Sweeney et al. 1995) on courtship latency and index, and female mating receptivity by using a single-pair mating paradigm (Lu et al. 2012). I found that blocking *Gr8a*-expressing sensory neurons in females leads to a higher sexual receptivity (willingness to mate) relative to control females when courted by wild-type males (**Figure 3A)**. Similarly, homozygous (Lee et al. 2012) and hemizygous (null *Gr8a* allele across a chromosomal deficiency) also show higher mating receptivity (**Figure 3B-C**). I next attempted to down-regulate *Gr8a* in a tissue specific manner using $Gr8a^{RNAi}$ obtained from the VDRC collection; however analysis of RNAi efficacy with RT-qPCR indicated that the $Gr8a^{RNAi}$ line was ineffective.

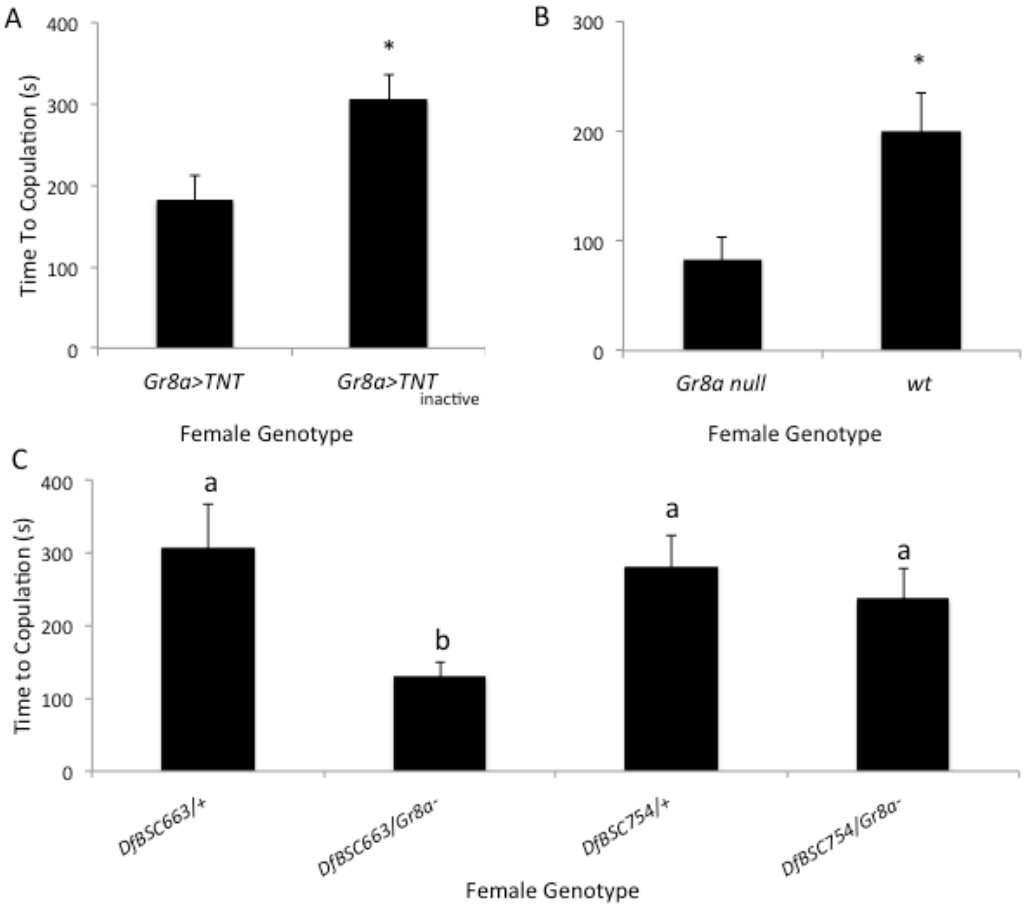

Figure 3. *Gr8a* is required for normal female sexual behaviors. (A-C) *Gr8a* mutant females are more receptive than wild-type *D.melanogaster* females. Letters indicate significance p<0.05 ANOVA (C), *, p<0.05, Mann Whitney Rank Sum Test (A,B).

1.2b Gr8a mutation has no effect on virgin male courtship behaviors

In contrast to females, I did not find a significant effect of blocking *Gr8a*-expressing neurons or the *Gr8a* mutant allele on virgin male mating behaviors as measured by courtship index or latency toward wild-type virgin females (**Figure 4A,B**). However, I did find that females are more attracted to *Gr8a* mutant males, indicating either a reduction in the level of inhibitory pheromone or the increase of attractive pheromone (**Figure 4C**). Since removal or silencing of the receptor in females makes them more receptive to copulation (**Figure 3**), it is

more parsimonious to assume that removing the receptor will result in lack of perception of an inhibitory signal, rather than making a female more sensitive to an attractive signal. Therefore it is likely that *Gr8a* is involved in the perception and production of an inhibitory signal.

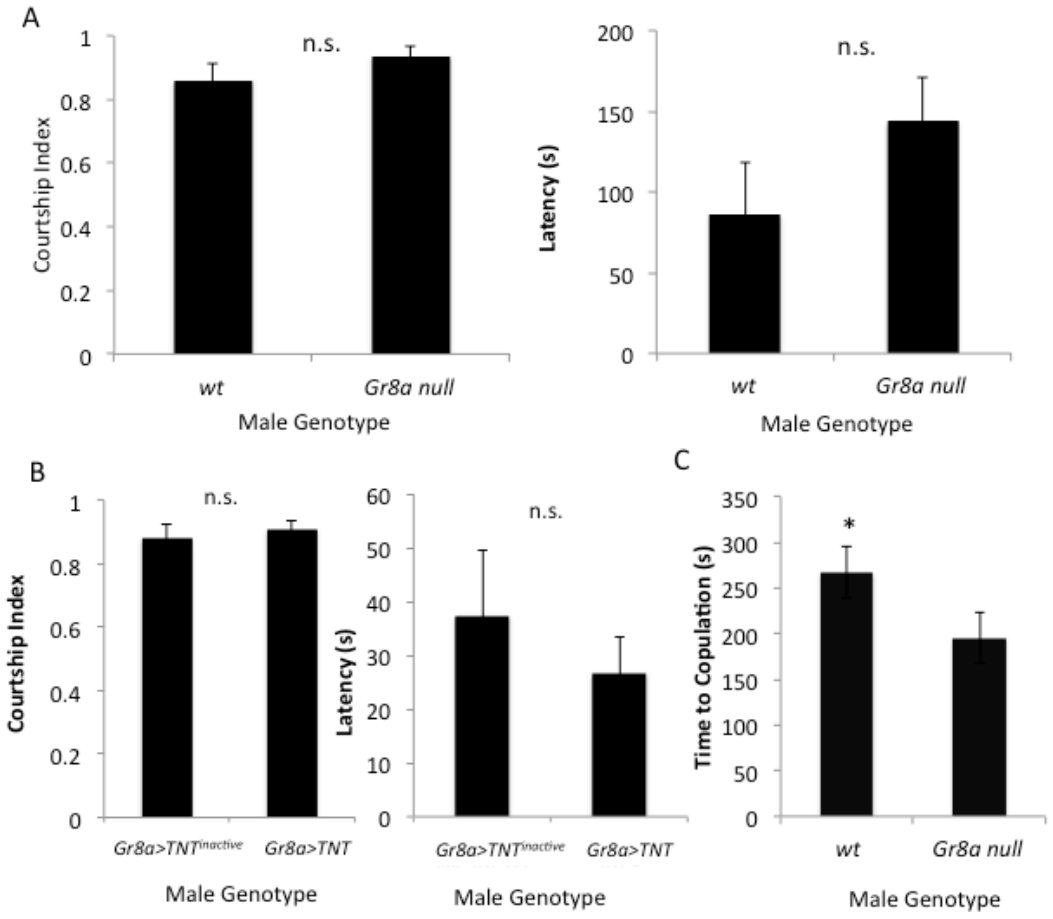

Figure 4. *Gr8a* mutants have no effect on male courtship index or latency toward wild-type females. (A) Courtship Index and Latency (s) of wild-type (*wt*) and *Gr8a null* males toward *wt* decapitated females. (B) Courtship Index and Latency (s) of control *Gr8a-gal4/UAS-IMP-TNT-V1A* ($TNT^{inactive}$) and *Gr8a-gal4/UAS-TNT-E* mutant males towards wild-type females. (C) *wt* female receptivity (time to copulation (s)) toward *wt* or *Gr8a null* mutant males. Letters indicate significance $p<0.05$ ANOVA (C,E), *, $p<0.05$, Mann Whitney Rank Sum Test, n.s.,non-significant ($p>0.05$).

1.2c Gr8a is required for normal male post-mating behaviors

Previous studies indicated that inhibitory mating pheromones are transferred from males to females during fruit fly copulation to suppress subsequent mating with other males (Yew et al. 2009; Zawistowski and Richmond 1986). Since my data suggest that mutant *Gr8a* males produce

lower levels of inhibitory pheromones than wild-type males, I hypothesized that females mated to *Gr8a null* males will be more attractive to wild type males than females mated to wild-type males because of a lower level of post-mating inhibitory pheromones. Indeed, I found that wild-type males find wild-type females that were previously mated with a *Gr8a null* male more attractive than females that first mated with a wild-type male (**Figure 5**). These data suggest that *Gr8a null* males do not transfer inhibitory mating pheromones to the female during copulation. However, we found no effect of the *Gr8a* mutation on male fitness as measured by eggs laid (**Figure 6**). These data indicate that the overall sperm quality and count are normal in *Gr8a null* males.

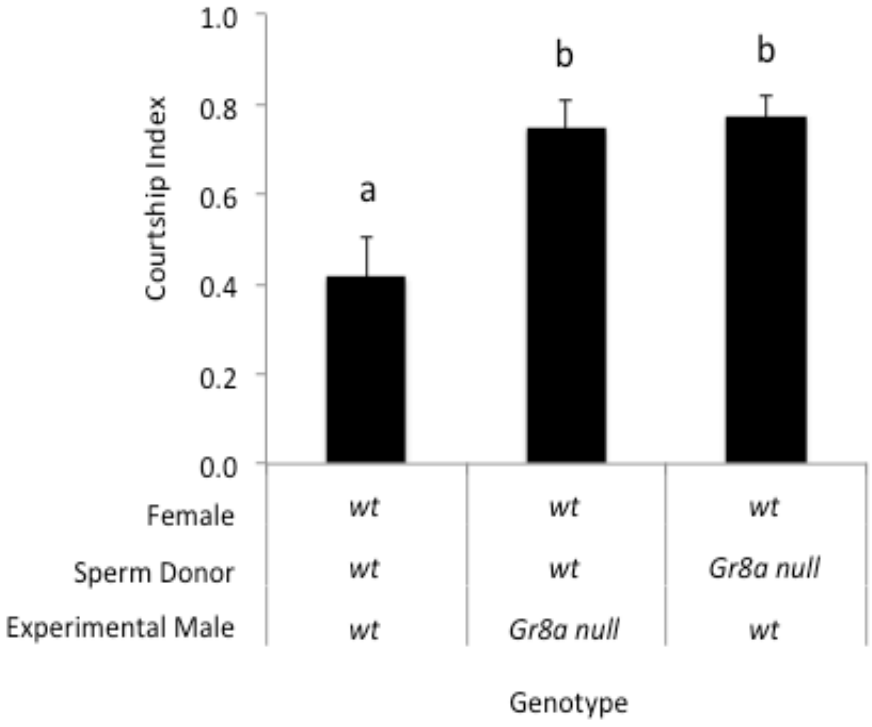

Figure 5. Sperm donor genotype affects mating behaviors of males, attractiveness of females. Sperm donor indicates male mated to female and experimental male indicates virgin male from which courtship index was measured. Letters indicate significance $p<0.05$ ANOVA.

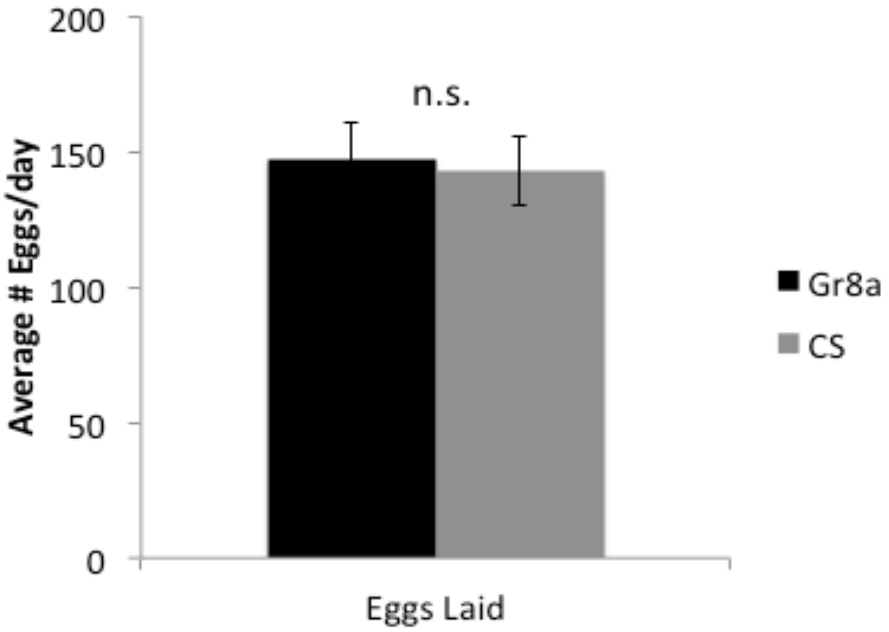

Figure 6. Female fitness does not change depending on mating male genotype. Average number of eggs laid per day by 5 females. Wild-type females mated with either wild-type (CS) or *Gr8a null* males.

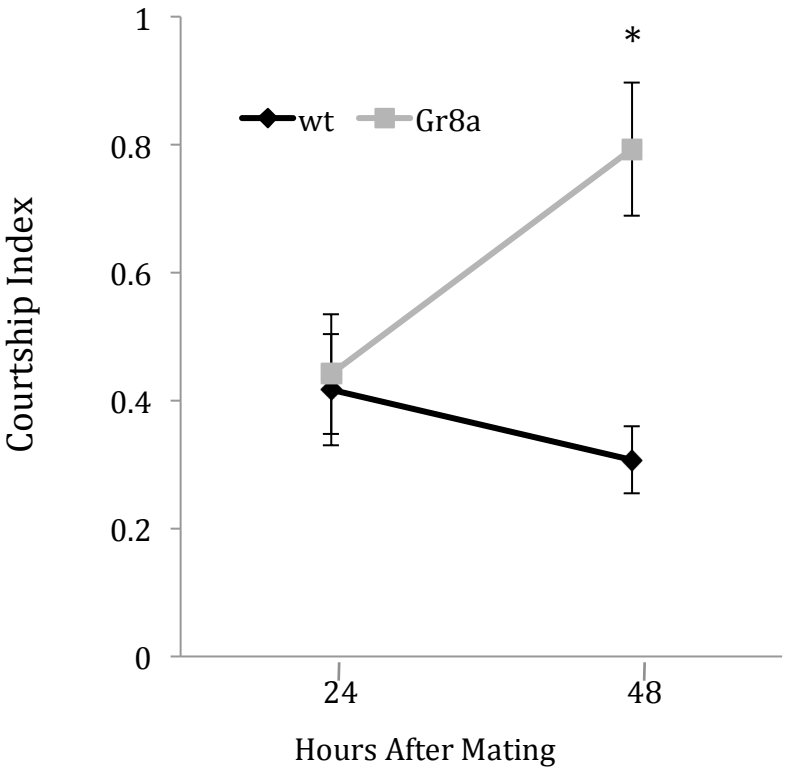

Figure 7. Mated *Gr8a null* females become attractive again after mating. *Gr8a null* mated females more attractive than wild-type females to wild-type males 48 hours but not 24 hours after mating. *Wild-type (wt)* and *Gr8a null* (*Gr8a*) females. *=$p<0.05$, ANOVA

To date, studies of *Drosophila* mating have claimed that males are the sole source of inhibitory pheromonal signals found in mated females (Chapman et al. 2003; Ram and Wolfner 2007; D. Scott and Jackson 1990; D. Scott, Richmond, and Carlson 1988; Yapici et al. 2008). Therefore, I anticipated that *Gr8a* mutant females that first mated with a wild type male would still be unattractive to wild type males. As predicted, wild type males find mated *Gr8a* mutant and wild type females equally less attractive 24 hours post-mating. Consistent with an abnormal ability to sense inhibitory mating signals, *Gr8a* mutant males could not recognize the mating status of females, which result in a high courtship index towards mated wild type females (**Figure 5**).

Typically, mated *Drosophila* females maintain their relative unattractiveness to other males 5-9 days post-mating (Tompkins and Hall 1981), which has been argued to represent a male-driven trait, functioning to increase male fitness by reducing the probability of repeat mating by females (Tram and Wolfner 1998). Surprisingly, I discovered that mated *Gr8a* mutant females become sexually attractive again to wild type males after the first 24h post mating (**Figure 7**). These data indicate that the post-mating reduced female attractiveness is a complex trait that is not solely driven by male fitness as is commonly described in the literature. Males transfer several compounds along with their seminal fluids during mating that reduce female attractiveness. However, my data indicate that up to 24h post-mating, the reduced attractiveness of females is not driven by male factors, but rather depends on *Gr8a* function via female-intrinsic processes. The ability of female flies to regulate their own attractiveness would be beneficial for the female in the case that the act of mating itself results in reduced fitness. Therefore, the putative gustatory receptor *Gr8a* appears to play a complex pleiotropic role in various aspects of sexual attractiveness and mating receptivity in both male and female flies.

Together, these behavioral data indicate that *Gr8a,* and the chemosensory neurons that express it, are playing a role in detecting inhibitory pheromones in both males and females, and the synthesis of an inhibitory pheromone in males. The observed pheonotypes are consistent with reduction in the preception and/or synthesis of an inhibitory signal rather than an increased sensitivity or synthesis of an excitatory signal since it is more likely that the perception of an inhibitory signal *Gr8a* detects will be hindered when the *Gr8a* receptor is removed, rather than a fly becoming more sensitive to a positive signal created by *Gr8a* when the receptor is removed.

1.3 *Gr8a and the cells that express it play a role in sex-related pheromonal signatures.*

To directly test the hypothesis that *Gr8a* plays a role in regulating the CHC profile via its action in oenocytes, I next examined the effects of the *Gr8a* mutation on the adult cuticular pheromone profile. Chemical analyses of adult CHCs from *Gr8a* mutant and wild type animals revealed that the mutation has a significant impact on the overall CHC profile in both males and females (**Figure 8, Tables 2 and 3).** Analyses of individual components of the CHC profile indicated that alkenes and methyl-branched CHCs are the most affected compounds by the *Gr8a* mutation (**Figure 8)**. Although a specific mating function for these CHCs has not been described yet for *D. melanogaster*, similar CHCs have recently been implicated in the evolution of mate choice and speciation in the related *D. serrata* (Chung et al. 2014). Although expression levels of *Gr8a* are significantly lower in the female abdomen, my data suggest that *Gr8a* plays a role in regulating the CHC profile in both sexes.

Because *Gr8a* mutant females regained their attractiveness 48 hours post-mating, we hypothesized that their pheromonal profiles will differ significantly from wild-type at 48 hours post-mating. Our results supported our hypothesis and we found that there are significant differences between *Gr8a* mutant and wild-type female CHC profiles 24 and 48 hours post-

mating (**Figure 9, Table 4**). Since *Gr8a* mutant females lose their inhibitory signature 48h post-mating, we hypothesized that *Gr8a* expression will increase in female oenocytes as a mechanism to induce a female specific post-mating inhibitory pheromone. However, we did not find an increase in *Gr8a* expression in virgin versus mated wild type females (**Figure 10A,B**).

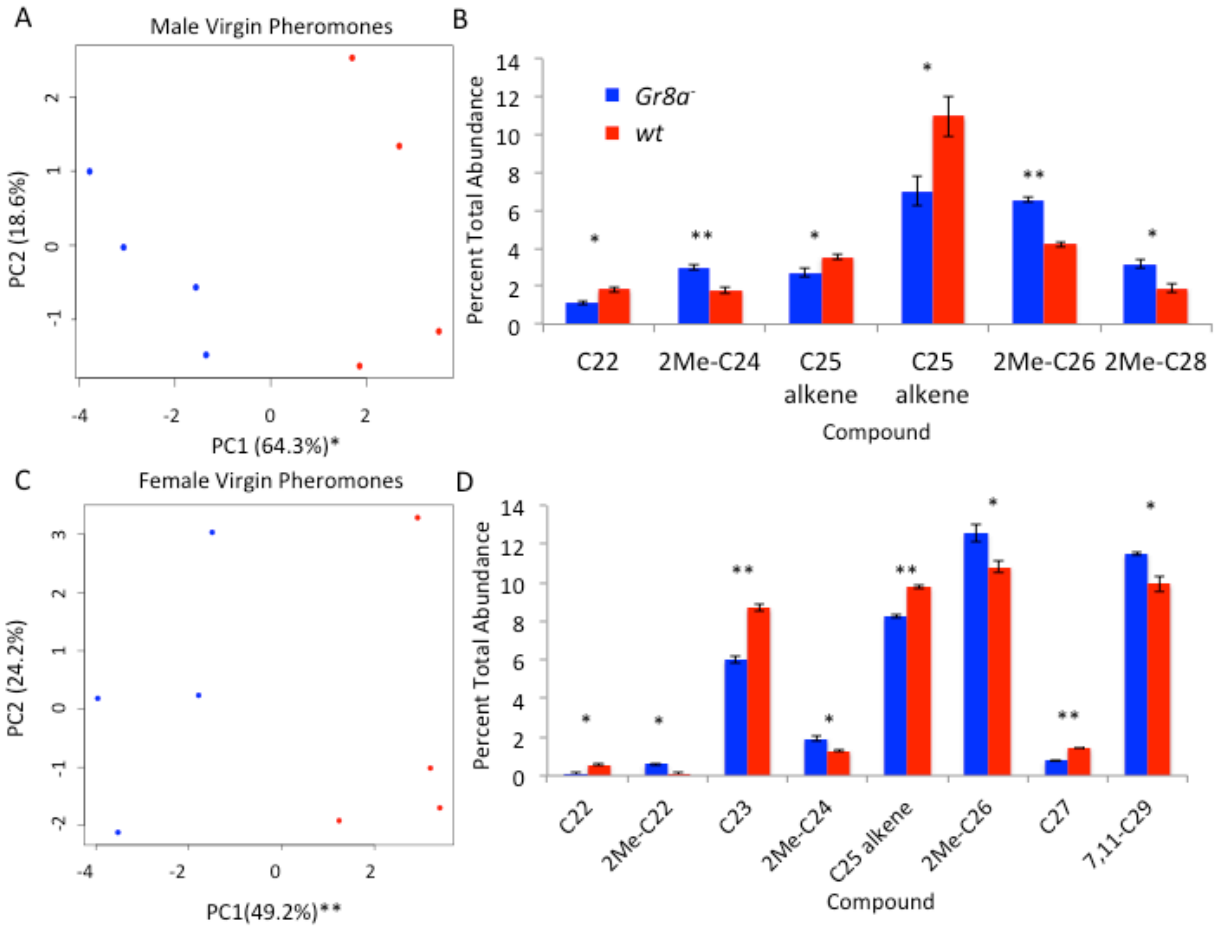

Figure 8. Pheromone profiles differ between *Gr8a* null mutants and wild-type (*CS*) flies. (A,C) PCA results for *Gr8a null* and wild-type (*wt*) virgin males (A), and virgin females (C). Blue circles represent *Gr8a null*, and red circles represent *wt* flies. (B,D,) Abundance of specific compounds for virgin males (B) and virgin females (D). Principle Components Analysis and MANOVA (A,C), Mann Whitney Rank Sum Test (B,D). *, $p<0.05$, **, $p<0.001$.

Further, because previous studies have shown that plasticity in the *Drosophila* CHC profile is driven by factors such as mating status and the social environment (Everaerts et al. 2010; Kent et al. 2008; Krupp et al. 2008), I also asked whether *Gr8a* expression might change in males in response to changes in the social environment. However, we did not observe any

changes in male *Gr8a* expression in association with change in social environment when the ratio of male to female fly was varied. (**Figure 10C**). Together, these data suggest that *Gr8a* plays an important role in regulating the post-mating pheromonal signature of females, but without affecting the post-mating behavioral switch (Chapman et al. 2003; Chen et al. 1988; Kubli 2003; Yang et al. 2009).

Table 2. Male Virgin Pheromones. % Total is percent total abundance. RT= retention time. + = increase in % total abundance, - = decrease in percent total abundance in *Gr8a* profile relative to wild-type. *= p<0.05 ns=non-significant

R.T.	Compound	*wt* % total	*Gr8a* % total	Change	Sig.
11.39	C21	1.109	1.028	-	ns
13.16	C22	1.822	1.142	-	*
13.315	unknown	0.478	0.538	+	ns
15.03	C23	17.706	14.628	-	*
15.27	7,11-C23 diene	46.690	49.383	+	*
15.4	unknown	4.089	3.563	-	*
15.57	cVA	5.379	6.918	+	ns
17.95	2Me-C24	1.770	2.981	+	*
18.84	C25 alkene	3.543	2.721	-	*
19	C25 alkene	10.971	7.022	-	*
21.66	2Me-C26	4.202	6.554	+	*
25.18	2Me-C28	1.879	3.180	+	*

Table 3. **Virgin Female Pheromones**. % Total is percent total abundance. RT= retention time. + = increase in % total abundance, - = decrease in percent total abundance in *Gr8a* profile relative to wild-type.

RT	Compound	*wt* % total	*Gr8a* % total	Change	Sig.
13.16	C22	0.568	0.000	-	*
14.2	2Me-C22	0.000	0.584	+	*
15.03	C23	8.707	6.034	-	*
15.19	C23 alkene	2.807	3.097	+	ns
15.269	unknown	0.910	0.870	-	ns
16.91	C24	0.554	0.000	-	*
17.95	2Me-C24	1.281	1.923	+	*
18.84	C25 alkene	9.790	8.265	-	*
19	C25 alkene	4.620	4.709	+	ns
19.07	7,11-C25 diene	5.068	4.925	-	ns
19.33	5,9-C25 diene	1.384	1.274	-	ns
21.66	2Me-C26	10.818	12.543	+	*
22.48	C27	1.433	0.794	-	*
22.55	C27 alkene	2.113	1.981	-	ns
22.72	C27 alkene	2.519	2.614	+	ns
22.82	7,11-C27 diene	32.140	33.077	+	ns
23.05	5,9-C27 diene	3.259	3.165	+	*
25.18	2Me-C28	2.753	3.130	+	ns
26.34	7,11-C29 diene	9.948	11.502	+	*

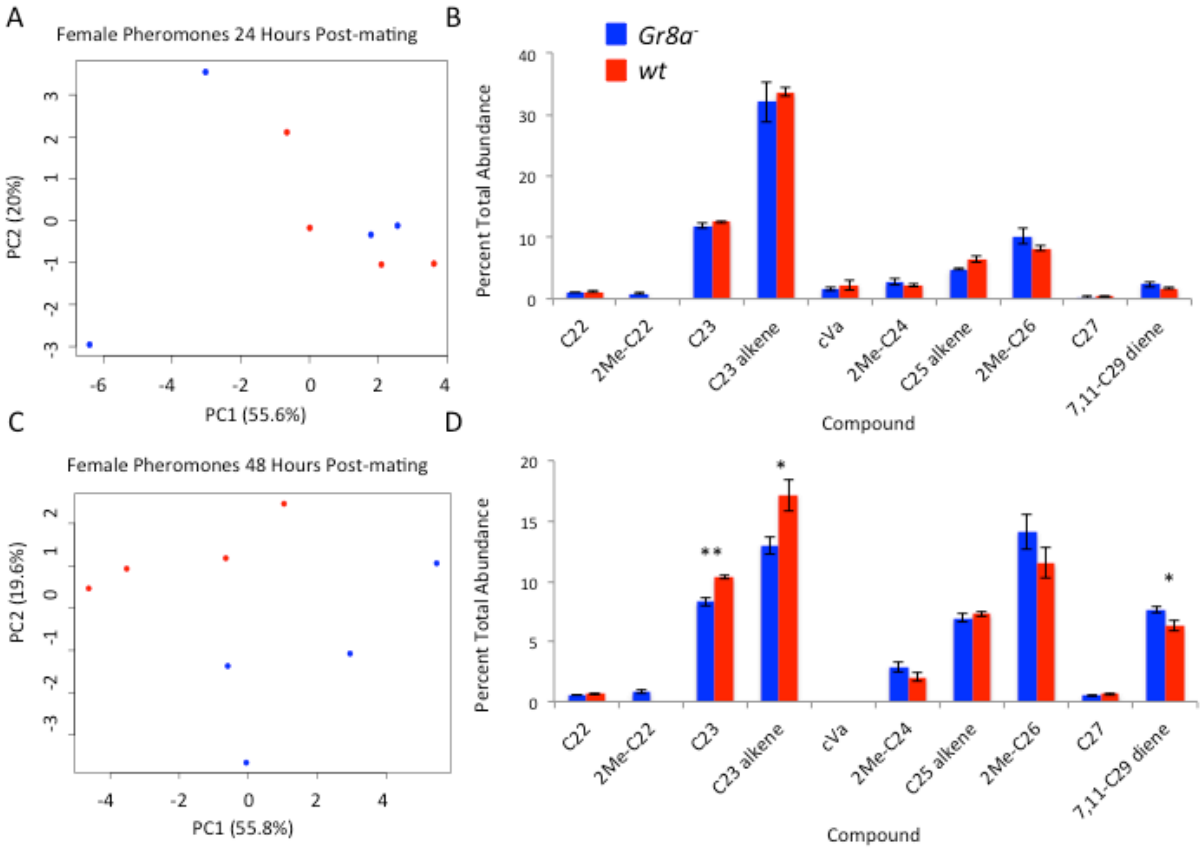

Figure 9. Pheromone profiles differ between mated female *Gr8a* null mutants and wild-type (*CS*) flies. (A,C) PCA results for *Gr8a null* and wild-type (*wt*) females 24 (A), and 48 (C) hours after mating. Blue circles represent *Gr8a null*, and red circles represent *wt* flies. (B,D,) Abundance of specific compounds for females 24 (B) and 48 (D) hours after mating. Principle Components Analysis and MANOVA (A,C), Mann Whitney Rank Sum Test (B,D). *, p<0.05, **, p<0.001.

Table 4. Mated Female Pheromones. % Total is percent total abundance. RT= retention time. + , increase in % total abundance, - , decrease in percent total abundance in *Gr8a* profile relative to wild-type, *, p<0.05, Mann Whitney rank sum test.

RT	Compound	**Wt 24h % total**	**Wt 48h % total**	Change	Sig.	**Gr8a 24h % total**	**Gr8a 48h % total**	Change	Sig.
11.4	C21	1.13	0.64	-	*	1.02	0.53	-	*
13.18	C22	1.14	0.62	-	*	1.05	0.55	-	*
13.34	C22 alkene	0.00	0.00	n/a	n/a	0.35	0.00	-	*
14.2	2Me-C22	0.00	0.00	n/a	n/a	0.86	0.81	-	n.s
15.05	C23	12.52	10.34	-	*	11.85	8.29	-	*
15.23	C23 alkene	33.63	17.16	-	*	32.02	13.00	-	*
15.32	C23 alkene	0.00	0.00	n/a	n/a	2.33	0.00	-	*
15.41	C23 alkene	3.21	1.57	-	*	3.53	1.09	-	*
15.59	CVA	2.24	0.00	-	*	1.50	0.00	-	*
16.92	C24	0.00	0.00	n/a	n/a	0.00	0.26	+	*
17.02	C24 alkene	0.28	0.00	-	*	0.22	0.00	-	*
17.96	2Me-C24	2.28	2.03	-	n.s.	2.72	2.91	+	n.s.
18.85	C25 alkene	6.43	7.31	+	n.s.	4.81	6.95	+	*
19	C25 alkene	6.83	5.16	-	*	8.06	4.89	-	*
19.07	7,11-C25 diene	3.78	4.47	+	*	3.05	3.50	+	n.s.
21.66	2Me-C26	8.17	11.53	+	*	10.19	14.09	+	n.s.
22.47	C27	0.40	0.64	+	*	0.27	0.53	+	*
22.54	C27 alkene	0.49	1.43	+	*	0.00	1.43	+	*
22.7	C 27 alkene	0.46	1.67	+	*	1.13	2.56	+	*
22.8	7,11-C27 diene	13.01	23.91	+	*	13.47	25.31	+	*
23.04	5,9-C27 diene	0.00	3.27	+	*	0.00	3.34	+	*
25.19	2Me-C28	3.07	2.22	-	n.s.	2.30	2.09	-	n.s.
26.34	7,11-C29 diene	1.74	6.26	+	*	2.39	7.67	+	*

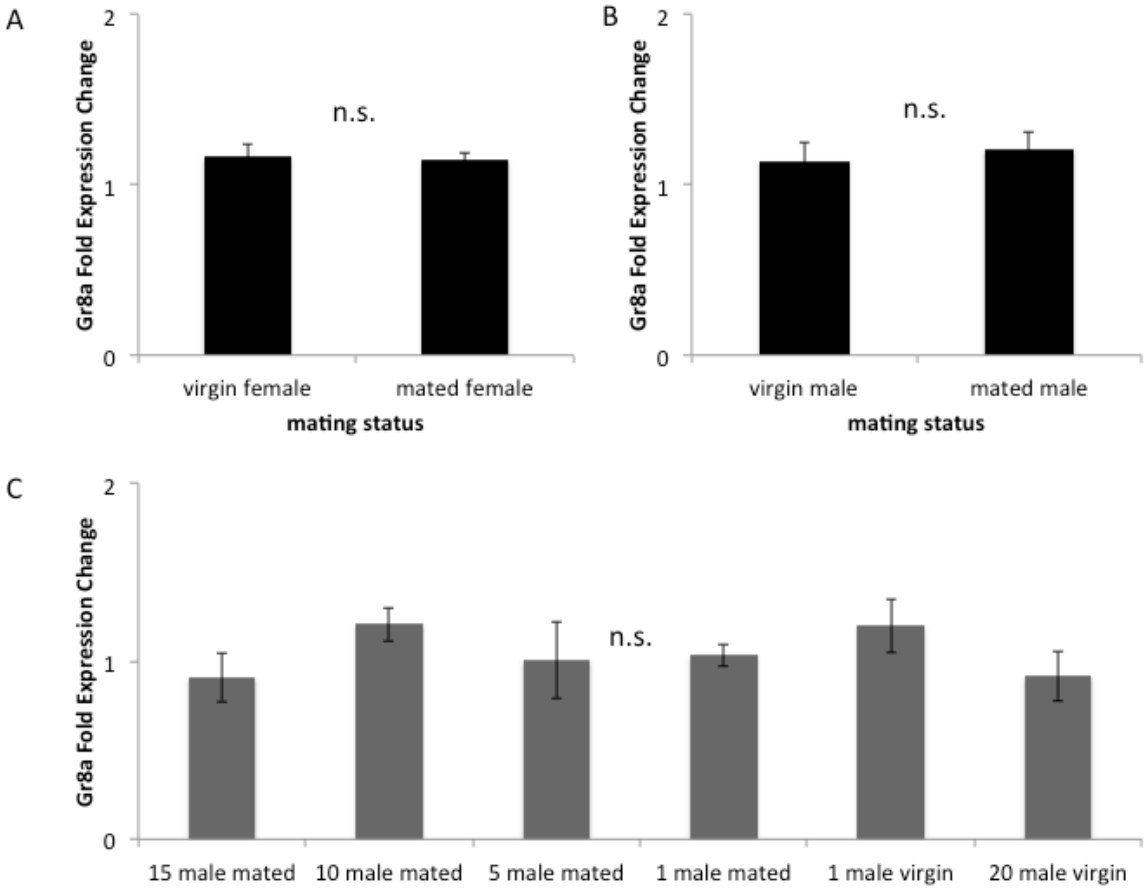

Figure 10. *Gr8a* expression does not change based on mating status or social context. A. *Gr8a* fold expression change by mating status-CS flies in females (left) and males (right). B. Male *Gr8a* fold expression change in differing social contexts. N=4. In C, mated males were kept in groups of 20 flies comprised of both sexes.

Discussion

Recent studies have suggested that some *Gr* genes contribute to pheromonal perception in flies (Bray and Amrein 2003; Moon et al. 2009; Watanabe et al. 2011). However, their role in pheromone synthesis has not been previously explored. Our data indicate that *Gr8a*, a sexually dimorphic member of the gustatory receptor family in *Drosophila melanogaster*, plays a role in mediating mating behaviors by affecting both the perception and synthesis of cuticular pheromones. Furthermore, *Gr8a* seems to play essential roles in both pre- and post-mating behaviors associated with reproduction in flies.

Gr8a is not only a neuronal chemoreceptor, but also contributes to pheromone production for one or multiple inhibitory pheromones. This is supported by our findings that *Gr8a null* females have higher sexual receptivity than wild type animals, and that *Gr8a* null males have increased attractiveness compared to wild-type males. In addition, our data indicate that *Gr8a* mutant males may not transfer inhibitory pheromones during copulation since mated females are more attractive to males when they have previously mated with a *Gr8a null* male than with a wild-type male. Further, although *Gr8a null* mutant female virgins are more receptive, *Gr8a null* mutant females are no more willing to re-mate than wild-type females. This indicates that *Gr8a* is involved in post-mating inhibitory pheromone synthesis in females without affecting the neurophysiological and behavioral changes associated with mating (Chapman et al. 2003; Chen et al. 1988; Kubli 2003; Yang et al. 2009).

Thus far, our data do not provide the precise identity of the *Gr8a* ligand. Nevertheless, our studies indicate that *Gr8a* may affect the overall quality of the pheromone profile in males (pre-mating) and females (post-mating). Our studies also imply that *Gr8a* contributes to the transfer and perception of inhibitory pheromones from males to females during copulation. Previous work showed that the male-specific inhibitory CHCs, cVA and CH503, are transferred to females during copulation (Vander Meer et al. 1986; Yew et al. 2009; Zawistowski and Richmond 1986). Pheromone analyses indicated that *Gr8a* mutation did not affect the synthesis or transfer of cVA (**Figure 8 and 9, Tables 2 and 4**). However, since cVA is a volatile, short-lived pheromone that is exclusively sensed by the olfactory system (P. Clyne et al. 1997; Ha and Smith 2006; Kurtovic, Widmer, and Dickson 2007) it is not likely a *Gr8a* ligand. In contrast, CH503 is a non-volatile, male-specific inhibitory pheromone that can be detected on the female

cuticle up to 10 days post-mating (Yew et al. 2009). Thus, it is plausible that CH503 and similar compounds are ligands of *Gr8a*.

In our model, GR8A functions in the perception of pheromones via gustatory receptor neurons, while its role in pheromonal composition may include several possibilities. As part of a single synthesis pathway, GR8A may directly or indirectly regulate a single step in the pathway via a positive or negative feedback loop. Though the gene may be functioning in both the perception and production of pheromones, it may be acting as a pheromone receptor in both of these, one serving in a signaling pathway and the other being cell-intrinsic. As pheromone production is a complicated process involving many different steps and enzymes, further studies would be required to elucidate the exact mechanism by which the receptor functions in pheromone production. Its involvement in a feedback loop is one of several likely possibilities.

Several studies have focused on the perception and production of pheromones as controlled by separate genes as they take place in different cells (Blows 1999; Butlin and Ritchie 1989; Kronforst et al. 2006; Lande 1981; Lofstedt et al. 1989; M G Ritchie 2000; Ryan 1988; K. L. Shaw and Lesnick 2009; Sureau and Ferveur 1999). However, this study indicates that it is possible for both the perception and production to be controlled by one pleiotropic gene, as in *Gr8a*. Though the exact mechanism by which this occurs requires further exploration, this study gives us a basis by which to understand how pheromone-receptor systems have evolved, also having implications for the maintenance of reproductive isolation between species via chemical communication. If true, our "pleiotropy model" stipulates that inter-species sequence variations in *Gr8a*- and related pheromone receptors orthologs are involved in the maintenance of reproductive barriers between closely related *Drosophila* species.

Methods

Fly Rearing and Strains. All flies were maintained on a standard cornmeal medium under a 12:12 light-dark cycle at 25 Celsius. *D. melanogaster Canton-S (CS)* served as our wild-type strain for most experiments. *D. melanogaster* w^{1118} wild-type flies were used only where indicated. *Gr8a-GAL4*, *UAS-Gr8a,* and *Gr8a null* allele were previously published (Lee et al. 2012; Weiss et al. 2011). Originally in the w^{1118} background, the *Gr8a null* mutant was outcrossed for six generations into the *CS* wild-type background. *UAS-TNT-E* and *UAS-TNT-IMP-V1-A* were obtained from C. O'Kane (Cambridge, England) and *PromE(800)-GAL4* and *PromE(800)-GAL4;luciferasedesat1* from Joel Levine. *Gr8a*^{RNAi} came from the Vienna *Drosophila* RNAi Center (http://stockcenter.vdrc.at).

***Gr8a* Expression and Immunohistochemistry.** To visualize expression patterns of *Gr8a* in males and females, progeny of the cross *Gr8a-GAL4* and *UAS-YC2.1* were obtained and aged 5 days. Whole flies were mounted onto slides and viewed using a Nikon-A1 confocal microscope. To visualize co-expression patterns of *Gr8a* and oenocytes in the abdomen, progeny of the cross *Gr8a-GAL4/cyo;luciferasedesat/TM3,Sb* and UAS-CD8::GFP were obtained and aged for 24 hours before dissections. Abdomens and brains were dissected and antibody stained according to previous protocols (Laissue et al. 1999; Wu and Luo 2006). Rabbit anti-GFP antibody and a secondary donkey anti-rabbit antibody coupled to Alexa Fluor 488 were used to visualize GFP. Mouse anti-luciferase and a secondary donkey anti-mouse antibody coupled to Alexa Fluor 568 was used to visualize luciferase. Ten samples of each sex were mounted in Vectashield mounting medium with DAPI (Vector Labratories, Inc.). All samples were viewed using a Nikon-A1 confocal microscope.

RNA and qPCR analysis. Virgin flies were separated and collected by sex under CO_2 and aged 5 days on standard cornmeal medium. They were then kept at -80°C until RNA extraction. To

separate body parts, microcentrifuge tubes were dipped in liquid nitrogen and then vortexed repeatedly until heads, appendages, and bodies were clearly divided. Total RNA was then extracted from tissues using the Trizol Reagent (Invitrogen). cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen) with 500 ng total RNA in a 20 uL reaction. Real-time quantitative RT-PCR was carried out as in previous publications. Gene-specific primers were designed on the IDT website (https://www.idtdna.com/site), and the housekeeping gene, *Rp49,* was used as a loading control as previously described by the Ben-Shahar lab (REFS).

Courtship Behavior. Virgin *D. melanogaster* wild-type and mutant male flies were collected upon eclosion under CO_2 anesthesia and kept separately in small vials (12 x 75mm). Virgin females of all species were collected upon eclosion and kept in groups of up to 10 flies in single-species vials. All vials contained standard cornmeal medium. Virgin males and females were aged 4-7 days under constant conditions of 25 degrees C and a 12:12 light-dark cycle before behavioral experiments to ensure all species' reproductive maturation. Flies were aspirated into circular arenas of 22 mm diameter and recorded for 10 minutes when assaying male behaviors and 15 minutes when assaying female behaviors. All behavioral assays were videotaped and analyzed as previously described (Ben-Shahar et al. 2007, 2010; Lu et al. 2012). For male behavior, latency and courtship index were recorded. We defined latency as the time lapsed until the male started to court the female. The courtship index is the amount of time he actually spends courting the female in the specified time period. The start of courtship was recorded by the first wing extension of the male. For female behavior, the time from the start of male courtship to copulation was recorded. Courtship experiments were performed under red light unless otherwise indicated.

Fertility Assays. Virgin male and female *D.melanogaster CS* and *Gr8a* mutant flies were collected upon eclosion as described above. On the third day post-eclosion, single pair matings were started in small vials (12 x 75mm) containing standard cornmeal medium. Twenty-four hours after mating, males and females were separated and males were discarded. Twenty-four hours after separation from the males, females were housed in groups of five in bottles with plates containing grape powder, agar and water. Females were allowed to lay eggs for 24 hours, after which the plates were removed, the eggs were counted, and new plates were placed in the bottles. The following day, the grape agar mixture containing larvae was carefully removed from each plate and placed into a vial with cornmeal medium. This process was repeated over five days. On day 17, all eclosed adult flies were counted.

Pheromone Analysis. Virgin flies were collected upon eclosion under a light CO_2 anesthesia and kept in single-sex vials in groups of 10 with 4 biological replications for each genotype and sex. Virgin flies were aged for 5 days on standard cornmeal medium at 25C. For collection of mated flies, both females and males were aged 3 days and then a single mating pair was placed in a small vial with standard cornmeal food for 24 hours. The pair was then separated for 24 hours before collection. On the morning of day 5, flies were transferred to foodless vials for one hour and then frozen in eppendorf tubes at -80C until analysis. Copulation was confirmed by the presence of larvae in the vial of mated flies several days later. Pheromone washes were performed by adding each group of 10 flies to 200 uL of hexane (Sigma-Aldrich #52766) in a 1.5 mL glass vial (Sun Sri #200 250) capped with Teflon-lined caps (Sun Sri #500 062). The flies were washed for five minutes. The hexane was then extracted from each vial with a glass pipette and stored in a 1.5 mL glass vial. For biological replications, groups of 10 flies were kept in single-sex vials were washed for 5 minutes in 200 uL of hexane. Cuticular hydrocarbons were

assayed by gas chromatography and mass spectroscopy, as previously described. Results were analyzed using Principle Components Analysis in R (Mardia, Kent, and Bibby 1979; Venables and Ripley 2002). Multivariate analysis of variance was completed on the first three principal components, which accounted for most of the variation between our groups.

CHAPTER 3: The contribution of pleiotropic chemoreceptors to pre-zygotic mating barriers

Introduction

Robust and reliable communication between animals during mating is important for maintaining species boundaries. Thus, all species utilize signals that signify important information to potential mates such as sex, age, mating status, and species identity (Chung and Carroll 2015; J.-F. Ferveur 2005; Wyatt 2003; Yew and Chung 2015). Theoretical and experimental studies have demonstrated that divergence in species-specific pheromones and their cognate receptors between populations may facilitate reproductive isolation, leading to speciation (Boake 1991; Butlin and Ritchie 1989; Chung and Carroll 2015; J. F. Ferveur and Sureau 1996; Leary et al. 2012; Smadja and Butlin 2009; Symonds and Elgar 2008). However, major gaps remain in the molecular and genetic understanding of how pheromonal systems evolved, and how they serve to maintain reproductive isolation between closely related species.

Drosophila species are found worldwide. Some species are allopatric in terms of geographical distributions and/or preferences for host plants (Markow and O'Grady 2005). However, many sympatric *Drosophila* species often exhibit robust abilities to discriminate between con- and heterospecific individuals, which is essential for preserving species mating boundaries, typically via pre-zygotic, mating preferences. Consequently, pheromonal communication during the courtship ritual is essential for the correct identification of potential mates, and thus represents a key pre-zygotic behavioral species barrier. Courtship in the *Drosophila* genus is a genetically-determined, innate behavior that comprises a specific sequence of behaviors in *D. melanogaster* males: orientation, tapping, wing extension and vibration, tasting, and attempting copulation, after which females may accept or reject the male by ovipositor extrusion, kicking, or flying away (Greenspan and Ferveur 2003; Lasbleiz, Ferveur,

and Everaerts 2006; O'Dell 2003). During courtship, a bouquet of pheromonal signals on the fly cuticle relay important information to each fly, including the age, sex, mating status, and species of the potential mate (Claude Antony and Jallon 1982; Billeter et al. 2009; Jallon and David 1987)

In the fly, cuticular hydrocarbons (CHCs) act as the main mating pheromones. CHCs are fatty-acid derived hydrocarbons that are synthesized in specialized abdominal subcuticular cells termed oenocytes (Billeter et al. 2009; Makki, Cinnamon, and Gould 2014). There is a great amount of quantitative and qualitative variability in pheromones across species, differing in the presence and location of double bonds, as well as the chain length. CHC's help to prevent desiccation, but often play an important pheromonal role in preserving reproductive behavioral barriers as they vary according to age, sex, diet, geographic origin, and species (Claude Antony and Jallon 1982; Billeter et al. 2009; Jallon and David 1987; Wigglesworth 1945). A handful of CHCs have been shown to influence behavior in *Drosophila*, including mate choice, male-to-male aggression and aggregation (J.-F. Ferveur 2005; Wicker-Thomas, Guenachi, and Keita 2009; Yew and Chung 2015). In *Drosophila melanogaster*, the male-specific pheromone 7-tricosene (7-T), functions as a female aphrodisiac, while the female-specific 7,11-heptacosadiene (7,11-HD) and 7,11-nonacosadiene (7,11-ND) act as male aphrodisiacs (Everaerts et al. 2010; J.-F. Ferveur 2005). In addition, male-specific pheromones, cVA and CH503, which are transferred to the female upon mating, serve as anti-aphrodisiacs to other males (Butterworth 1969; Ejima 2015; Jallon 1984; Yew et al. 2009). However, there are currently no known pheromonal functions for the majority of CHCs in *Drosophila*. When pheromones are ablated, species boundaries are lost, highlighting their importance for reproductive isolation (Billeter et al. 2009).

However, the specific roles of many pheromones on the *Drosophila* cuticle and their receptors remain unknown (Everaerts et al. 2010; Yew and Chung 2015).

Several multigene families encoding chemosensory receptors are important for chemical communication in *Drosophila*: olfactory receptors (Or's), gustatory receptors (Gr's), ionotropic receptors (Ir's), and DEG/ENaC channels (Hanukoglu and Hanukoglu 2016; Zelle et al. 2013). These receptors function in detecting food odors and compounds as well as receiving pheromones during interactions between individuals (Montell 2009). During courtship, *Drosophila* may sense both volatile and contact (non-volatile) pheromones. While volatile pheromones are used to support medium range attraction and orientation, contact pheromones are used during the "tapping" and "licking" steps and carry specific information such as species and sex. During courtship when the males "tap" the females, contact pheromones are sensed via chemosensory receptors, such as Gr's, in their forelegs to gather important information about the status and species of the female.

Gr's in *D.melanogaster* are composed of a diverse family of 60 genes encoding 68 alternatively spliced seven-transmembrane receptors (P. J. Clyne 2000; Dunipace et al. 2001; Robertson, Warr, and Carlson 2003; K. Scott et al. 2001). Expression patterns of family members include sensilla in the proboscis, legs, and anterior margins of the wings and been implicated in sensing bitter and sweet compounds as well as pheromones, yet the function of many Gr's remain unknown (Dahanukar et al. 2007; Jiao et al. 2008; Jiao, Moon, and Montell 2007; Lee, Moon, and Montell 2009; Moon et al. 2006, 2009; Slone, Daniels, and Amrein 2007). Few Gr family members have been found in atypical tissues as well and might have non-chemosensory functions. Recent studies indicate that gustatory receptors function as heterotrimeric receptors (Poudel et al. 2015; Shim et al. 2015).

In insects, the perception and synthesis of pheromones are mediated by different cell types and depend on very different molecular pathways. Consequently, it is typically assumed that signal and receiver are coded by different genes, which may or may not be genetically coupled (Blows 1999; Butlin and Ritchie 1989; Kronforst et al. 2006; Lande 1981; Lofstedt et al. 1989; M G Ritchie 2000; Ryan 1988; K. L. Shaw and Lesnick 2009; Sureau and Ferveur 1999). However, in Chapter Two, I demonstrated that in *Drosophila*, pheromone perception and production can be linked via the action of pleiotropic gustatory receptors. I established that *Gr8a*, a gustatory receptor gene, is involved in the regulation of female mating decisions, possibly via the perception and production of methylated CHCs, which are known to have functions in mating behaviors in other *Drosophila* species (Chung et al. 2014). With this information, I then hypothesized that due to the effects of pleiotropic *Gr8a* on the production and perception of pheromones, replacing endogenous expression with orthologs would cause behavioral mating barriers. To test this, I transgenically expressed the *Gr8a* ortholog from *D.mojavensis* as well as the endogenous *D.melanogaster Gr8a* in the *D.melanogaster* genetic background and observed its effect on pre-zygotic isolation between the two transgenic lines. Such pleiotropic loci could represent a simple evolutionary solution to the conundrum of how species-specific pheromone-dependent mating barriers evolve and maintain mating barriers between closely related species.

Results

The results presented in Chapter Two of my thesis indicate that *Gr8a* plays a role in both sensory perception and pheromone production and is sexually dimorphic in *Drosophila melanogaster*. As sexually dimorphic genes are often a result of sexual selection and can have implications for behavioral isolation (Panhuis et al. 2001), we then asked if a similar expression pattern exists across the *Drosophila* clade. We found that, as in *D. melanogaster*, *Gr8a*

expression is significantly higher in males relative to females in all other *Drosophila* species tested (**Figure 1A,B**). Comparative analysis of the GR8A protein sequence revealed a domain of high inter-species variability, which may represent the ligand-binding pocket (**Figure 1C**). However, the homologous region in the other sexually dimorphic abdominal gustatory receptor proteins did not show a similar pattern of high variability, which may suggest that this variability is not functionally important or is unique to the functions carried by *Gr8a* (**Table 1, Figure 2**).

If GR8A functions in the maintenance of species boundaries, there may be signatures of selection among GR8A and orthologs. The branch-site test for selection indicates that variations in the *Gr8a* sequence across the *Drosophila* genus have been shaped by episodic diversifying selection in the phylogenetic tree at the branch leading to the following species: *D. virilis, D. persimilis, D. pseudoobscura, D. mojavensis, D. willistoni* and *D. grimshawi* (**Figure 3**). The ratio of nonsynonymous to synonymous mutations, dN/dS, or ω, signifies negative purifying selection (ω<1), neutral mutations (ω =1), or positive diversifying selection (ω >1). The ω value inferred for positively selected sites along this branch was ω=31. Other branches tested were not characterized by significant selection (**Table 2**). In the context of my primary hypothesis, evidence of positive selection may indicate the importance of GR8A sequence for maintaining species boundaries.

As the pheromonal bouquet of each species is very different, ligand-binding properties of specific pheromone receptors may be important for detecting species-specific pheromone ligands. We found that the CHC profiles of *Gr8a null* males and females differ significantly from wild-type flies in several compounds (Chapter 2); therefore we hypothesized that pleiotropic *Gr8a* could be sufficient to maintain species-specific mating barriers. To test my hypothesis I generated *D.melanogaster* transgenic strains that express either the endogenous or a

heterospecific *Gr8a* gene and examined the effects of this genetic manipulation on assortative mating (Castrezana and Markow 2008). To specifically examine the role of *Gr8a* in chemosensory-dependent mating barriers, I first asked which *Drosophila* species with an available sequenced genome has the most robust chemically-induced mating barrier relative to *D.melanogaster*. Using cross-species male-female mating assays, and mate-choice assays (**Figures 1C, 4**), I identified the *Gr8a* cDNA from *D.mojavensis* as the best candidate. The diversity of the GR8A amino acid alignment between *D.melanogaster* and *D.mojavensis* further supported it as a strong candidate for transgenic expression in *D. melanogaster* (**Figure 1**). In our behavioral assays, male *D.melanogaster,* with or without vision (i.e., under white or red light conditions, (Boll 2002)) showed very low courtship towards *D.mojavensis* females, and also made very few courtship errors when presented with *D.mojavensis* females. This indicates that the behavioral barrier between *D.melanogaster* males and *D.mojavensis* females is robust, making *D.mojavensis Gr8a* an ideal candidate gene to make our transgenic line. In addition, selection experiments indicated episodic diversifying selection for a group of *Drosophila* including *D.mojavensis*, further making it a great candidate to test our hypothesis that GR8A may function in the maintenance of species behavioral isolation (**Figure 3**). However, we did not observe any significant assortative mating among the transgenic flies (**Table 3**). During single pair encounters, males and females with heterospecific and conspecific *Gr8a* cDNA had a similar latency to court, courtship index, and time to copulation (**Figure 5**).

Further, as *Gr8a null* virgin females are more receptive towards conspecific males (Chapter 2), we hypothesized that they would also be more receptive towards heterospecific males. Could having a *Gr8a null* mutation increase a female's receptivity towards heterospecific males, dissolving the pre-zygotic behavioral barrier between them? Using the males of different

Drosophila species, I tested whether or not *D.melanogaster* females with a *Gr8a null* mutation were more receptive than wild-type females to these heterospecific males. However, in our behavioral assays no courtship was observed by any of the heterospecific males towards the *D. melanogaster* wild-type or *Gr8a null* mutant females (data not shown). Therefore, it remains unclear whether or not *Gr8a null* mutant females would be more receptive than wild-type females to heterospecific males and whether or not a *Gr8a null* mutation in the female fly would be sufficient to decrease behavioral isolation between species.

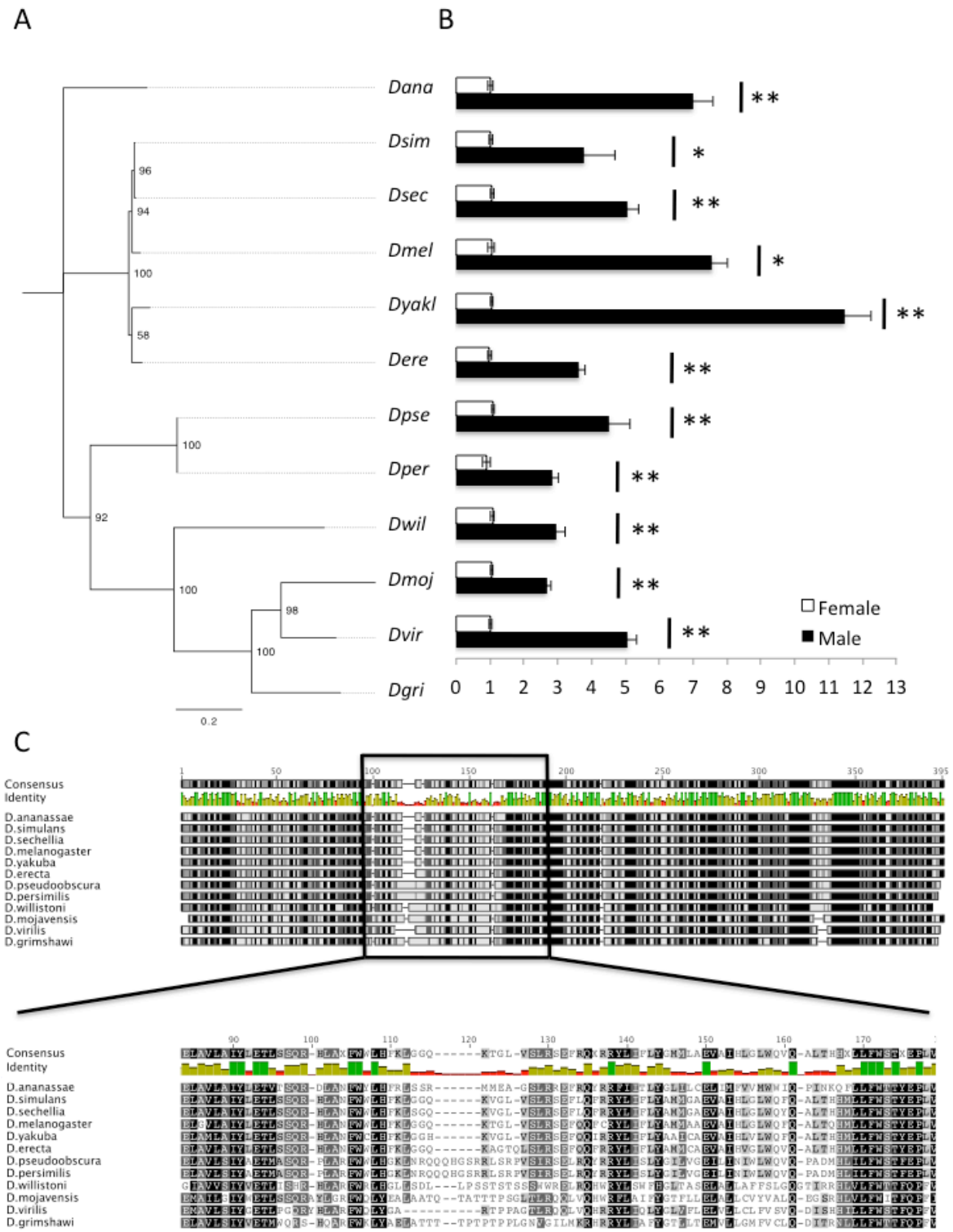

Figure 1. *Gr8a* expression is sexually dimorphic in 11 *Drosophila* species. (A) Phylogenetic tree of *Gr8a* and orthologs. Substitution rate = 0.2. (B) Fold change in *Gr8a* expression between sexes in 11 *Drosophila* species. N=4, Black bars, males; Gray bars, females. Bars correspond to species listed to the left. *, $p<0.05$; **,$p<0.001$ (Mann Whitney Rank Sum Test). *Drosophila grimshawi* is absent from our qPCR data as they were not available from Bloomington Stock Center. (C) Multiply-aligned amino acid sequences of *D.melanogaster Gr8a* (*CG15371*) and

homologs. Note area of less conservation between amino acids 100-170. Numbers on top of alignment indicate amino acid number. Black, 100% identical; Dark Gray, 80-100% similar; Light Gray, 60-80% similar, White, less than 60% similar (Blosum62 score matrix, threshold=1).

Table 1. *Gr* genes with sexually dimorphic expression in the abdomen. +, PCR product present, -, PCR product absent.

	Mated Male	**Virgin Male**	**Mated Female**	**Virgin Female**
GR2a	-	-	+	+
GR8a	+	+	-	-
GR36c	+	+	-	-
GR63a	+	+	-	-
GR64a	+	+	-	-
GR64d	+	+	-	-
GR98b	-	-	+	+

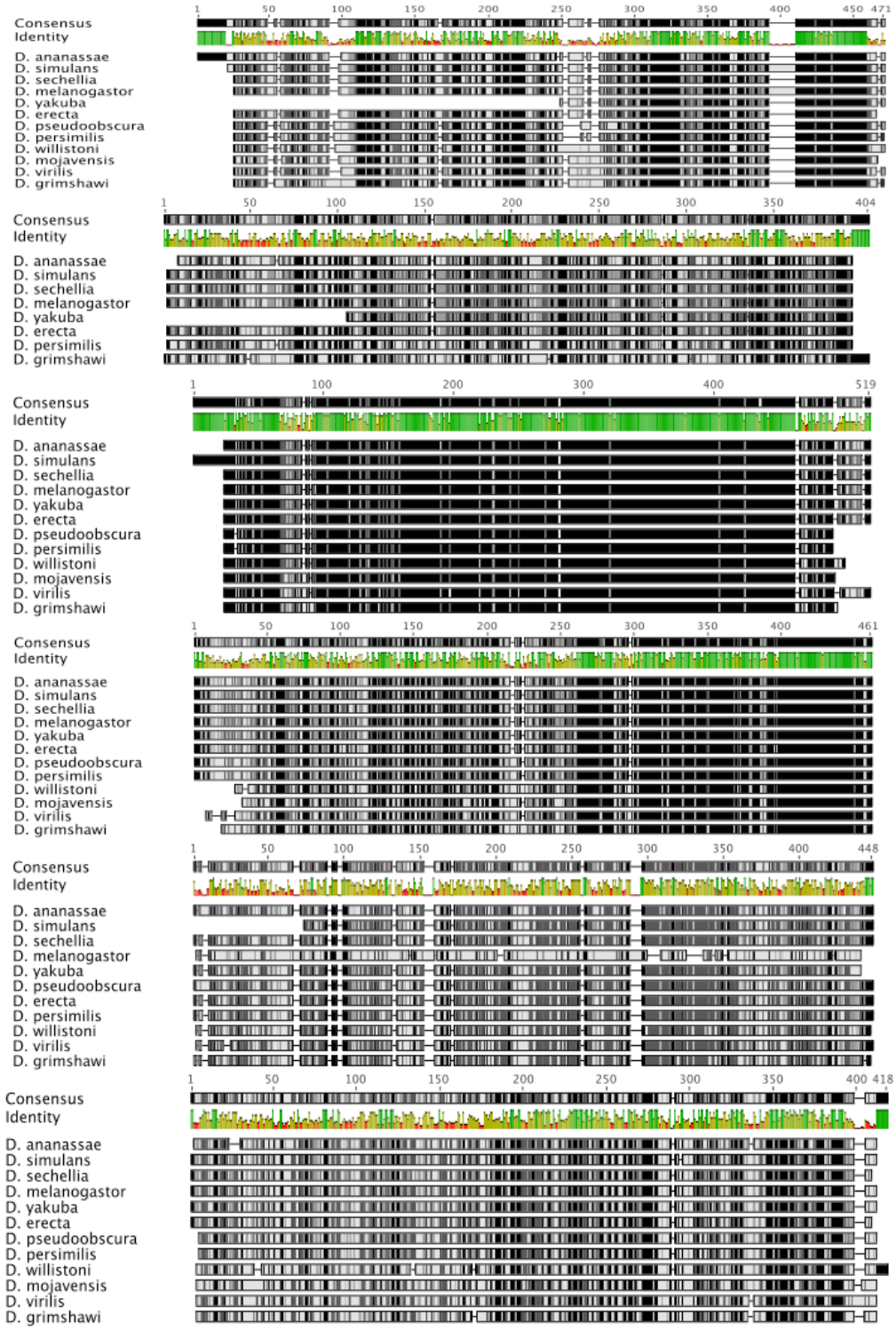

Figure 2. Sexually dimorphic *Gr's* have variable protein sequences across species . Multiply-aligned amino acid sequences of *D.melanogaster Gr8a* (*CG15371*) and orthologs. Top to bottom (*D.mel* gene name) *Gr2A*, *Gr36c*, *Gr63a*, *Gr64a*, *Gr64d*, *Gr98b*. All species-specific gene names can be found in **Appendix 1**. Numbers on top of alignment indicate amino acid number. Black, 100% identical; Dark Gray, 80-100% similar; Light Gray, 60-80% similar, White, less than 60% similar (Blosum62 score matrix, threshold=1)

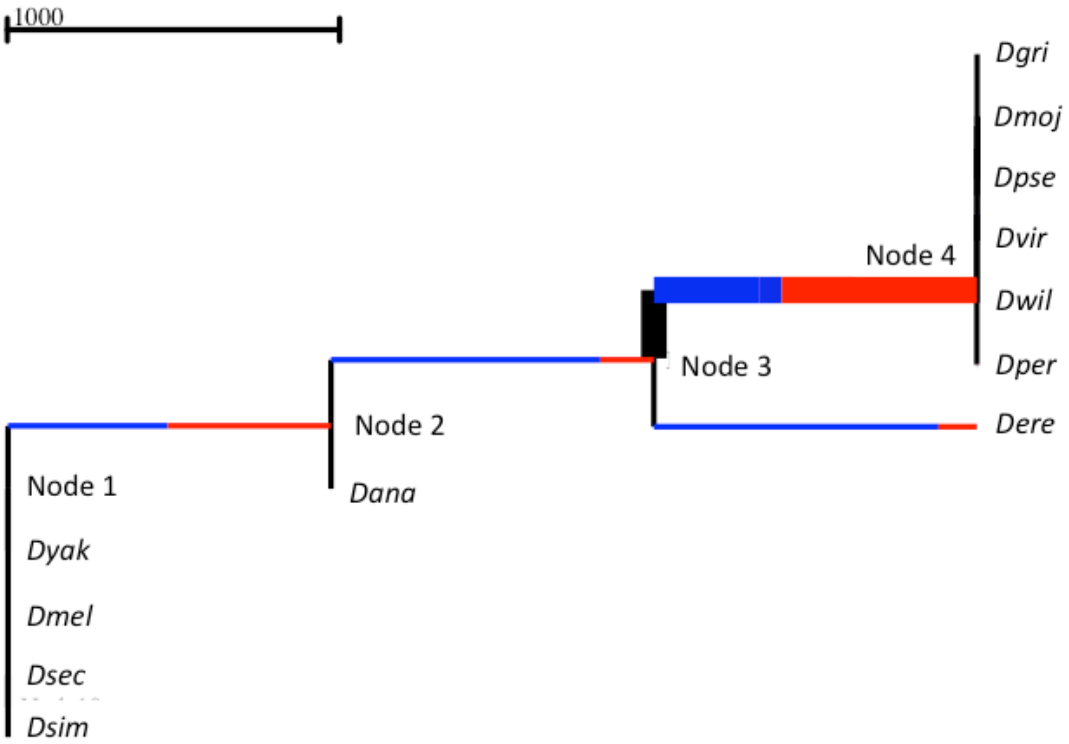

Figure 3. GR8A is under episodic diversifying selection. Neighbor joining tree of GR8A and ortholog protein sequences from 12 species with sequenced genomes that represent the major phylogenetic clades of *Drosophila*. The branch at Node 4 has been classified as undergoing episodic diversifying selection by the sequential likelihood ratio test at $p<0.05$. The corrected p-value for episodic selection of the branch at Node 4 is 0.013, corrected for multiple testing using the Holm-Bonferroni method. Color corresponds to the strength of selection: Red to $\omega>5$, blue to $\omega=0$ and black to $\omega=1$. The width of each color bar indicates the proportion of sites in the corresponding class. Scale bar is the expected number of substitutions/nucleotide.

Table 2. Inferred branch-specific distributions of site-wise ω show evidence for episodic diversifying selection. ω^+= the ω value inferred for positively selected sites along each branch, Pr[ω= ω^+] = maximum likelihood estimate of the proportion of sites evolving at ω^+. Corrected p-value is the p-value corrected for multiple testing using the Holm-Bonferroni method. Significant branches highlighted in red. Branches are ordered in decreasing level of support for diversifying selection.

Branch	ω^+	Pr[ω=ω^+]	P-value	Corrected P-value
Node 4	**31**	**0.6**	**0**	**0.01**
Dmoj	24.64	0.77	0.01	0.2
Dere	10000	0.12	0.01	0.25
Dper	660.21	0.51	0.02	0.39
Dgri	10000	0.23	0.06	0.98
Node 1	6.17	0.01	0.24	1
Dmel	1.86	0.1	0.34	1
Dana	2.51	0.02	0.49	1
Dvir	1541.95	0.55	0.5	1
Dwil	33.47	0.1	0.5	1
Node 3	35.49	0.17	0.5	1
Node 2	37.49	0.51	0.5	1
Dyak	3.06	0.15	0.5	1
Dpse	0	1	1	1
Dsec	0	1	1	1
Dsim	0.1	0.06	1	1

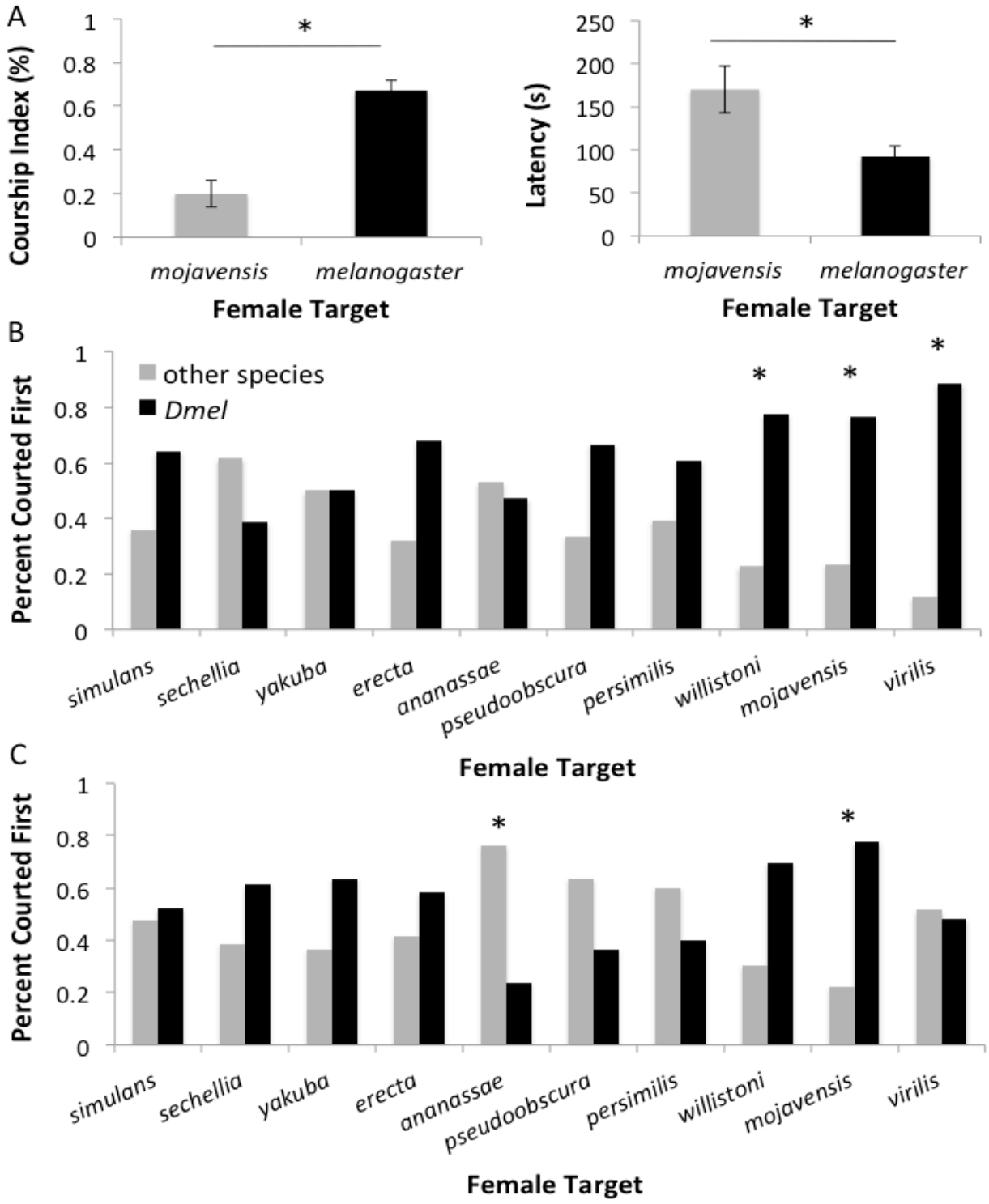

Figure 4. *D. mojavensis* females are not attractive to *D.melanogaster* males. Courtship index and latency of *D.melanogaster* males towards *D.melanogaster* and *D.mojavensis* females (A). Percent of target female species courted first in choice experiments by *D.melanogaster* males in white light (B) and red light (C). Gray bars, heterospecific female, black bars, *D.melanogaster* female. *, $p<0.05$, χ^2 test.

Table 3. Assortative mating experiment results for flies transgenic for *D.melanogaster* and *D.mojavensis Gr8a*. Female x Male. $Gr8a^{D.mel}$ =expresses *D.melanogaster Gr8a* cDNA in *Gr8a null* background, $Gr8a^{D.moj}$ = expresses *D.mojavensis Gr8a* cDNA in *Gr8a null* background.

Total # matings	$Gr8a^{D.mel}$ x $Gr8a^{D.mel}$	$Gr8a^{D.mel}$ x $Gr8a^{D.moj}$	$Gr8a^{D.moj}$ x $Gr8a^{D.mel}$	$Gr8a^{D.moj}$ x $Gr8a^{D.moj}$	X^2	p-value
165	43	42	44	36	.939	.816

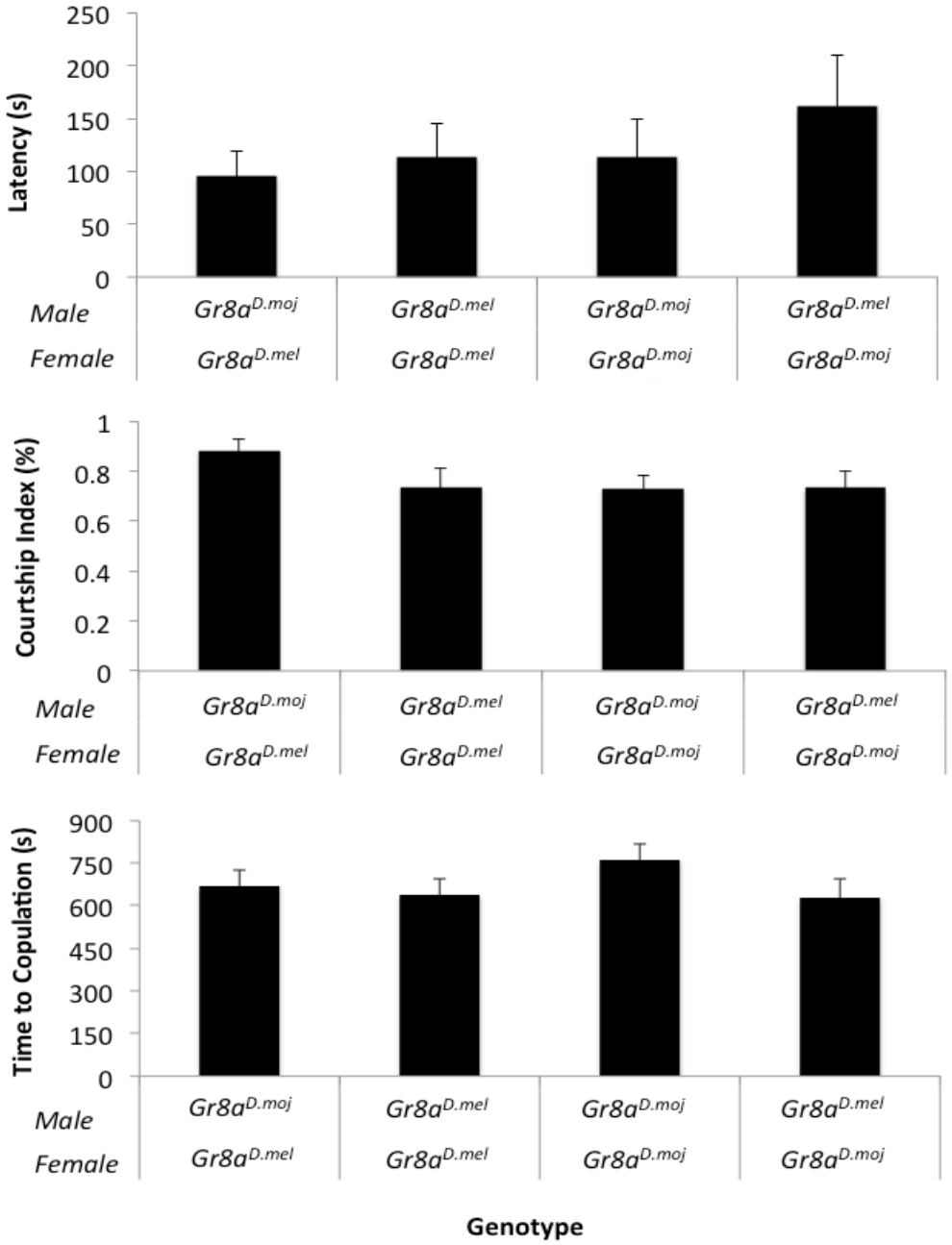

Figure 5. *Drosophila melanogaster* transgenic for *Gr8a* show no altered courtship behaviors. (A) Latency of transgenic males to courtship of transgenic females. (B) Courtship Index of transgenic males toward transgenic females. (C) Receptivity of transgenic females when courted by transgenic males. Genotypes: $Gr8a^{D.mel}$= *Gr8a null*;*UAS-Gr8a (D.mel)*/*Gr8a-Gal4*. $Gr8a^{D.moj}$ = *Gr8a null*;*UAS-Gr8a (D.moj)*/*Gr8a-Gal4*. Non-significant p>0.05, ANOVA, N=14-20.

Discussion

Pheromonal communication is imperative for the maintenance of pre-zygotic species barriers in many organisms. However, the genetic architecture and evolution of mating

communication systems represents an evolutionary puzzle as theory suggests that any changes in either signal or receptor would be selected against. My study tests a novel hypothesis that some chemosensory receptor genes play a dual role in both sensing pheromones and regulating their synthesis. Our data from Chapter Two suggest that mutations in a single gene could affect both signal and receptor while maintaining their functional coupling. These findings support a simple molecular solution to a complex evolutionary problem and indicate that behavioral barriers between closely related species can be maintained via a pleiotropic gene, perhaps having implications for speciation.

Theoretical and empirical studies have supported the idea that sexual communication systems can contribute to reproductive isolation and speciation through sexual selection (Barraclough, Harvey, and Nee 1995; Butlin and Ritchie 2009; Gray and Cade 2000; Panhuis et al. 2001; Smadja and Butlin 2009). However, with the exception of auditory communication in crickets (K. L. Shaw and Lesnick 2009), the genetic architecture of many of these communication systems remains unknown (Boake 1991; Butlin and Ritchie 1989; Hunt et al. 2012). Our work indicates that expressing the *Gr8a* cDNA of *D.mojavensis* in *D.melanogaster* is not sufficient to affect courtship behaviors or assortative mating in our behavior assays. Though I did not observe any atypical courtship phenotypes in our transgenic lines, this does not mean *Gr8a* has no effect on behavior. It is possible that the effect of *Gr8a* is simply not robust enough for our specific measured courtship parameters. The hypothesis that contact pheromones and their cognate receptors are important for the maintenance of behavioral barriers between closely related species remains plausible as courtship decisions are complex and are driven by multiple sensory modalities and pheromone receptors. Select gustatory receptors have recently been shown to act in groups rather than on their own (Jiao et al. 2008; Poudel et al. 2015). It is

possible that eliminating specific groups of endogenous gustatory receptors or expressing heterospecific groups of receptors rather than only eliminating one would result in a more robust phenotype.

Previous studies indicate GR8A is a receptor for the insecticide L-canavanine (Lee et al. 2012). Though *Gr8a* is expressed in sensilla in the proboscis, likely playing a role in feeding behaviors, it is also expressed in sensilla in the pre-tarsus of the forelegs (Chapter 2), suggesting a role in mating behaviors (Lu et al. 2012). It is likely that GR8A is also a receptor for inhibitory compounds, including those present in pheromonal profiles. Because I found a hyper-variable region in the *Gr8a* species alignment, I hypothesized that this may be a ligand-binding area. The recently identified CH503 may be one candidate compound for GR8A due to its inhibitory effect on behavior as well as its ability to remain on the female cuticle for several days (Yew et al. 2009). Further studies would need to be completed to confirm any inhibitory compounds GR8A binds in *D.melanogaster* and also extended to other species.

Uncovering the genetic architecture of chemical communication is important for determining the factors that contribute to reproductive isolation between species. As a widely used form of chemical communication during mating, pheromone diversity can contribute to the divergence or maintenance of species such as moths and *Drosophila* (Etges and Jackson 2001; Smadja and Butlin 2009). As mate recognition systems must be extremely specified, theory suggests there should be strong selective pressures against any changes in either the signal or receiver. A handful of studies show that divergence in mate recognition signals can be due to few or single genes of large effect, or a collection of genes of small effect (Blows 1999; Butlin and Ritchie 1989; Kronforst et al. 2006; Lande 1981; Lofstedt et al. 1989; M G Ritchie 2000; Ryan 1988; K. Shaw et al. 2011; K. L. Shaw and Lesnick 2009; Sureau and Ferveur 1999). Pleiotropy

in chemical communication could serve as a more parsimonious explanation for reproductive isolation between species, though there is little empirical evidence available (Bousquet et al. 2012; Butlin and Ritchie 1989; Marcillac, Grosjean, and Ferveur 2005; K. Shaw et al. 2011; Smadja and Butlin 2009). Studies on incipient species may be most informative as after a speciation event many other changes will have accumulated that are not important for isolation. As genome sequence data becomes available in a wide range of species, new studies can begin to uncover the roles of specific pheromones as well as the enzymes and receptors involved in pheromone production and identification. Scientists can then begin to develop a more complete understanding of how the diversity of species evolved and remain isolated.

Methods

Fly Rearing and Strains. All flies were maintained on a standard cornmeal medium under a 12:12 light-dark cycle at 25 Celsius. *D. melanogaster Canton-S (CS)* served as my wild-type background strain for all experiments. *Gr8a-GAL4* and *Gr8a null* allele were previously published (Lee et al. 2012). Originally in the w^{1118} background, the *Gr8a null* was outcrossed for six generations into the *CS* wild-type background. Species were obtained from the San Diego Stock Center (https://stockcenter.ucsd.edu), including *D. simulans* 14011-0251.192, *D. sechellia* 14021-0248.03, *D. yakuba* 14021-0261.01, *D. erecta* 14021-0224.00, *D. ananassae* 14024-0371.16, *D. pseudoobscura* 14011-0121.104, D. *persimilis* 14011-0111.50, *D. willistoni* 14030-0811.35, *D. mojavensis* 15081-1352.23, and *D. virilis* 15010-1051.118. The specific fly stocks were chosen based on the lines that were originally used for genome sequencing. They cover all the major clades across the *Drosophila* lineage.

Transgenic flies were generated using standard molecular methods and the ΦC31integrase system (Groth 2004). *Gr8a* sequences of *D. melanogaster* and *D. mojavensis* were synthesized to include restriction sites for EcoRI and NotI using GeneArt (Life Technologies). Both *D.melanogaster* and *D.mojavensis UAS-Gr8a*^{CDNA} lines were transformed into a *Gr8a null* background, resulting in complete substitution of the endogenous *Gr8a* with expression of a *Gr8a* ortholog.

Phylogenetic Analysis. *Drosophila melanogaster Gr8a* and orthologous protein sequences were mined in FlyBase and multiply-aligned using Clustal Omega (Sievers et al. 2011). The alignment was entered into ProtTest v 2.4 to determine the best model of protein evolution. From the Akaike and Bayesian information criterion scores (Abascal, Zardoya, and Posada 2005; Drummond and Strimmer 2001; Guindon and Gascuel 2003) we selected the appropriate substitution matrix. We then used a maximum likelihood approach and rapid bootstrapping within RAxML v 7.2.8 Black Box (Stamatakis 2006; Stamatakis, Hoover, and Rougemont 2008), on the Cipres web portal (Miller, Pfeiffer, and Schwartz 2010) to generate a phylogenetic tree. Visualizations of the bipartition files were made using FigTree v 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/). Alignments of GR's were visualized using MUSCLE in Geneious version 9.0.5 created by Biomatters, available from http://www.geneious.com.

Behavioral Assays. I performed assortative mating assays in chambers based on Castrezana and Markow 2008 and manufactured by the machine shop at Washington University in Saint Louis. Because my transgenic animals are of the same species, a fluorescent dust of different colors from Magruder Color Co (Alameda, CA) was used as a species marker. The flies were dusted and allowed 24 hours to clean themselves in a vial before behavioral experiments proceeded. This dust was found to have no effect on behavior (Castrezana and Markow 2008). Genotypes of

flies were *Gr8a null;Gr8a-Gal4/UAS-Gr8a*$^{D.mojavensis}$ or *Gr8a null;Gr8a-Gal4/UAS-Gr8a*$^{D.melanogaster}$. Ten flies of each sex and genotype (40 flies total) were aspirated into assortative mating chambers in white light. Total number of matings for each genotypic combination were recorded for 30 minutes, N=20.

Male choice experiments were performed in round courtship arenas. Briefly, one *D. melanogaster* virgin female and one interspecific virgin female was decapitated under CO_2 and placed in the arena. One virgin male *D.melanogaster* was then aspirated into the arena and behavior was video recorded for 10 minutes. The first female courted (by male wing extension) was noted. Courtship assays were performed under normal light conditions unless otherwise indicated and repeated (N=20-35) for each species.

Male no-choice experiments were performed in arenas of 22mm diameter. Males and females were separately aspirated into mating chambers and behavior was recorded for 10 minutes. No-choice experiments were conducted in red light.

Quantitative Expression Studies. Five day old flies of both sexes were collected upon light anesthesia and RNA was extracted using TRIZOL. cDNA was then synthesized with Superscript II with 500ng total RNA in a 20uL reaction. Real time quantitative RT-PCR was then performed as in previous publications (Lu et al. 2012). All gene-specific qPCR primers were made through IDT (https://www.idtdna.com/site) and species-specific *rp49* was used as a loading control. All qPCR primer sequences for *D.melanogaster Gr8a*, *Rp49* and orthologs can be found below.

Species	***Gr8a* Forward Primer**	***Gr8a* Reverse Primer**
D. melanogaster	TGACCATCAACATACGCATCG	CGTATATGAAGGCGGGAATCTC
D. simulans	GAACTTTTCGCTGCAACTCC	ACTTCGGTATAAACTGGATGGTG
D. sechellia	GAGATTCCCGCCTTCATATACG	GAGTTGCAGCGAAAAGTTCTG
D. erecta	CAGATTCAGAACTTTTCGCTGC	GGTGTAGATCATGTAGGTGCC
D. yakuba	TGCCTCGGACTAACAATTCTG	GTGTAGATCATGTAGGTGCCC
D. ananassae	AATGTACCGAAGTTTCCAGGG	GCGGGTATGATCAGGAAATAGTC
D. pseudoobscura	CCCGTTTCCGTGACAATATTG	ACCATCTACATATCCGTTGCC
D. persimilis	TTTCGCTTCTCCACACTGAC	AGGCGGGCAATATCAAAGAG

D. willistoni	GAAATGTTGCCCAGAATAGCC	CCCAAAGCATGTATAACCACTG
D. virilis	TCTTCAGATCCAAAACTTTTCGC	TTGGGCATCAGTTGTACGG
D. mojavensis	CATATACCCGCCTTTCTCTACAC	GTTCGTGCAGAATTTGTAGCG
Species	***Rp49* Forward Primer**	***Rp49* Reverse Primer**
D. melanogaster	ATCTTGGGCCTGTATGCTG	TGTGATGGGAATTCGTGGG
D. simulans	GTCGGATCGATATGCTAAGCTG	CAGATACTGTCCCTTGAAGCG
D. sechellia	CATACAGGCCCAAGATCGTG	CAGATACTGTCCCTTGAAGCG
D. erecta	GTCGGATCGATATGCTAAGCTG	CAGATACTGTCCCTTGAAGCG
D. yakuba	CATACAGGCCCAAGATCGTG	GGCATCAGATACTGTCCCTTG
D. ananassae	TACAGGCCCAAGATCGTTAAG	GTACTGACCCTTGAAGCGAC
D. pseudoobscura	CCAGCTCCAAAATGACGATTC	TCAATACCCTTAGGCTTGCG
D. persimilis	AAGCACTTCATCCGTCACC	TCAATACCCTTAGGCTTGCG
D. willistoni	AAGCACTTCATCCGTCACC	GTTGGGCATCAGATATTGGC
D. virilis	AGTCGGATCGTTATGCTAAGTTG	TGGAGGGTACGCTTGTTTG
D. mojavensis	ACCATTCGTCCAGCATACAG	TTGGCCCTTGAAGCGAC

Tests for Positive Selection. Protein sequences of *D.melanogaster Gr8a* and orthologs were mined in FlyBase (Flybase.org). They were then tested for specific codons under positive selection using the Branch-site Random Effects Likelihood (REL) test, available in the HYPHY package (Pond and Frost 2005) and accessed through Datamonkey website. Sites under positive selection were those sites with greater nonsynonymous (dN) than synonymous (dS) substitution rates (dN>dS) with significant p-values. The ratio of dN/dS, also called ω, signifies negative purifying selection (ω <1), neutral mutations (ω =1), or positive diversifying selection (ω >1). P-values were corrected for multiple testing using the Holm-Bonferroni method, and the p-values for all tests are shown in Table 2.

CHAPTER 4: Conclusions

How robust, species-specific chemical communication systems are maintained at the molecular and genetic levels is still an unresolved, fundamental problem in evolutionary biology. Due to the high specificity in the mate recognition system, the genetic components must be under strong selective pressures as a change in either will only be beneficial if a new signal is recognizable and relays information to a potential mate, or a new receptor recognizes an old signal. If the new signal is not recognizable, or a change in receptor renders it unable to perceive a current sexual signal, it will likely be eliminated by stabilizing selection (Brooks et al. 2005). Limited evidence supports a correlation between separate genes controlling signal and receiver (Kronforst et al. 2006; Wiley, Ellison, and Shaw 2012), but a more parsimonious genetic explanation for the maintenance of a functional signal-receiver in sexual communication and reproductive isolation between species is that signal and receiver are influenced by changes in a single pleiotropic gene (Bousquet et al. 2012; Fukamachi et al. 2009; Marcillac, Grosjean, and Ferveur 2005). My study provides one solution to how changes in a pheromone profiles (the signal) can be coupled to changes in a gustatory receptor (the receiver). I tested the novel hypothesis that some chemosensory receptor genes play a dual role in both sensing pheromones and regulating their synthesis. I suggest that mutations in a single chemosensory gene could affect both signal and receptor while maintaining their functional coupling. My findings support a simple molecular solution to a complex evolutionary problem and imply that behavioral barriers between closely related species could be maintained via a pleiotropic gene, perhaps having implications for speciation as well.

Gr8a and Chemoreceptor Pleiotropy

Often, tissues involved in pheromone production and perception are very different, and therefore pleiotropy may seem unlikely. However, my study indicated atypical expression of a group of gustatory receptors (Gr's) in the *Drosophila* abdomen. Because expression of *Gr8a* was discovered in the oenocytes, we hypothesized that it plays a role in pheromone production. This atypical expression is consistent with recent studies that also discovered atypical expression and functions for gustatory receptors (Jones et al. 2007; Miyamoto and Amrein 2014; Montell 2013; Ni et al. 2013; Thorne and Amrein 2008). To my knowledge, my study is the first to show that a mutant Gr gene affects the pheromone profile of *Drosophila melanogaster*, opening a new avenue for future research as atypical expression of *Gr8a* was found in the oenocytes.

While a number of quantitative changes were seen in the pheromone profiles of mutant versus wild-type flies, more information is needed to determine which compound acts as a ligand for *Gr8a*. Behavioral results suggest that *Gr8a* detects inhibitory compounds. This is consistent with studies showing that *Gr8a* detects the inhibitory compound *L-canavanine* (Lee et al. 2012; Shim et al. 2015). However *L-canvanine* is found in many legumes, few of which may be host plants for *Drosophila*. Therefore, it is highly likely that there is a different natural ligand to which *Gr8a* binds. As mutant *Gr8a* females are more sexually receptive, it is likely that *Gr8a* detects an inhibitory pheromone as they are often used during sexual behaviors. Perhaps mutant females are not able to detect an inhibitory compound being produced by the male. Further, mutant males find previously mated wild-type females attractive. Typically, mated females are not attractive to males and so this finding suggests males are not detecting post-mating inhibitory compounds either produced by the female post-mating or from the male that transferred compounds to the female. Lastly, females mated with mutant males remain attractive to wild-

type males, suggesting mutant males are not transferring an inhibitory pheromone. Due to this collection of mutant behaviors, it is likely that *Gr8a* detects an inhibitory pheromone.

A strong candidate for a *Gr8a*-binding ligand could be the male-specific inhibitory compound of CH503 as it inhibits sexual behavior in males across species and remains on the female cuticle for several days after mating (Ng et al. 2014; Yew et al. 2009; Yew and Chung 2015). However, the synthesis of this compound is difficult due to its large size and was unattainable at the time of this study. While cVa is also an inhibitory compound transferred from male to female during mating, cVa does not maintain inhibitory effects in the females for more than several hours and there is no consensus as to whether or not it actually moves from the reproductive tract to the cuticle of the female (Ejima 2015; Ejima et al. 2007; Vander Meer et al. 1986; Wang and Anderson 2010). cVa has been found to be detected by the olfactory system as well (Kurtovic, Widmer, and Dickson 2007), making it an unlikely candidate as the ligand for *Gr8a*. Further, we saw small changes in several compounds in mutant *Gr8a* pheromone profiles, including methyl-branched CHC's (mbCHC's). Due to recent evidence for their role in sexual behaviors, these may appear to serve as good candidates for *Gr8a* ligands as females discriminate between males with normal levels of methylated CHCs and males with altered levels. However, based on the results from the study by Chung *et al*., mbCHC's seem to act as aphrodisiacs (Chung et al. 2014), while my study and others (Lee et al. 2012; Shim et al. 2015) indicate *Gr8a* binds inhibitory compounds. Though the most abundant *Drosophila* CHC's have been well-studied, more clarity is needed for the behavioral effects of the majority of compounds in the pheromonal bouquet to determine a likely candidate ligand for *Gr8a.* Future studies involving perfuming CHC-less flies with synthesized compounds and measuring effects on sexual behaviors may shed light on this.

The mechanism by which *GR8A* functions in pheromone production in the oenocytes remains unclear at this time. A single gene, in theory, can have different functions due to alternative transcripts, tissue-specific expression, or developmental changes in expression to name a few. The single gene could also be performing similar functions but acting in different pathways in different tissues. One plausible mechanism by which the *GR8A* receptor may function is via a negative or positive feedback loop in oenocytes, detecting the amount of a specific pheromone and regulating the rate of its production. In this hypothesis, the GR8A protein acts as a receptor in both the sensory system (gustatory sensilla) to detect pheromones of a potential mate as well as cell-intrinsically as a receptor to regulate pheromone production in oenocytes. Future studies would be required to elucidate the mechanism by which *Gr8a* acts as a pleiotropic gene affecting pheromone production and perception.

Gr8a and Pre-zygotic Behavioral Isolation

Because *Gr8a* may function in both sexual receptivity in females and pheromone production, it could have implications for reproductive isolation, as chemical communication is often important for the behavioral isolation of species. Further, there is a hyper-variable region in the *Gr8a* amino acid sequence of different *Drosophila* species, possibly serving as a species-specific ligand-binding area. However, assortative mating was not observed between flies transgenic for *D.melanogaster* and *D.mojavensis Gr8a* cDNA. It is possible that mutation or transgenic expression of only one gene is not sufficient to produce robust courtship behaviors, which are likely the result of expression by a multitude of genes. This contradicts the hypothesis that a pleiotropic gene can affect the production and perception of a chemical signal. Recent studies indicate that Gr's together form a functional ion channel, and perhaps it is in working

with these other genes that sexual isolation is affected. A study by Shim *et al.* suggested that *Gr8a* forms a functional channel with *Gr66a* and *Gr98b* (Shim et al. 2015). Perhaps if all three of these genes were mutated then one might be more likely to see a measurable effect on male behavior or assortative mating. However, multiple gustatory receptors as well as other pheromone receptors, such as the ppk DEG/ENAC family (Hanukoglu and Hanukoglu 2016; Lu et al. 2012; Zelle et al. 2013), have been reported to have functions in pheromone detection and courtship behaviors, highlighting the possible role of multiple genes in addition to *Gr's* in behavioral isolation between species.

Chapter Three highlights the presence of *Gr8a* orthologs across *Drosophila* species (**Figure 1**). My data indicate that *Gr8a* plays a role in female sexual behaviors in *D.melanogaster*, and therefore it is possible that a mutation or deletion of *Gr8a* orthologs, rather than its replacement with the *D.melanogaster Gr8a* cDNA, may also have a measurable phenotypic effect in females of other species. Further, if *Gr8a* arose after speciation events, it may function differently based on the species. It will be useful moving forward to isolate specific genetic factors in each species with roles in sexual behavioral in order to more completely understand pre-zygotic isolation across *Drosophila* species.

As sexual isolation is species specific, and while *Gr8a* does not appear to be sufficient for behavioral isolation between *D.mojavensis* and *D.melanogaster*, this does not eliminate the possibility that the gene is important for maintaining barriers between other species. My positive results indicate that *Gr8a* is involved in the mate recognition system, influencing isolation, but may not be the driver of its evolution. Further studies on the importance of specific sensory modalities for the courtship between each species will shed light on the types of genes important for isolation. My study indicated vision is important for the isolation between male

D.melanogaster and female *D.virilis*-when vision was taken away males began courting randomly. However for other species' females, such as *D.mojavensis*, when vision was taken away males maintained inhibition towards females. This suggests that pheromones and chemosensory receptors may be more important for maintaining inhibitory effects between certain species. Perhaps when it comes to the different species of *Drosophila*, one must not generalize, but look species-specifically for the genetic basis of reproductive isolation between them. It is generally accepted that multiple sensory systems are involved in mate recognition and preference phenotypes, though the relative importance of each different sensory modality during courtship is complex and unclear (Krstic, Boll, and Noll 2009). In some species, chemosensation may be the most important sense to detect potential mates, while in others, auditory or visual senses may be imperative. For species encounters in which gustatory input is most important, for example, a mutation in *Gr8a* or other gustatory receptors may have a more robust effect on courtship and assortative mating. Further, it is possible that the genetic architecture underlying each of the modes of communication is different. One modality may require few genes of large effect while another may be under polygenic control via genes of small effect. Therefore, if multiple sensory modalities are at play in mate preference or signal phenotype, and each of these differs in underlying genetic architecture, there may exist a complex genetic underpinning, explaining why no robust assortative mating behaviors where observed when only one gustatory receptor, *Gr8a*, was mutated.

Further, one must consider that species may be isolated not behaviorally, but by other forms of pre-zygotic isolation such as habitat, location, host plant/substrate preferences, and zygote incompatibility as well as post-zygotic mechanisms such as hybrid sterility and unviability. Though we see a behavioral phenotype during courtship for *Gr8a null* mutants,

indicating it may have a role in pre-zygotic isolation, there are likely other isolating mechanisms at work, such as those listed above, in which *Gr8a* is not sufficient to hinder isolation between the species. There may also exist a combination of pre-zygotic isolation and post-zygotic isolation between populations (Nanda and Singh 2012). In some cases, reproductive isolation may only be a by-product of genetic divergence caused by environmental differences or strong genetic drift. There is also a possibility that traits involved in reproductive isolation have evolved due to ecological selection on phenotypes producing traits that become co-opted in mating behaviors, dubbed "magic traits" (Servedio et al. 2011; Thibert-Plante and Gavrilets 2013). This thesis does not neglect that there are multiple forms of isolation that can keep species separated, as well as multiple ways the traits involved can evolve. However, in many cases sexual selection and reinforcement are likely to generate genetic divergence between species and help to maintain isolation between them (Panhuis et al. 2001; Schluter 2001; Seddon et al. 2013). Therefore, as I found that *Gr8a* functions in female sexual behaviors, it is a plausible hypothesis that this pleiotropic gene could help to maintain sexual barriers between species.

The *Drosophila* genus currently contains over two thousand known species and *Drosophila melanogaster* is a model organism in genetics and evolutionary biology due to its simple genetics, short lifespan, and genetic manipulability. Many *Drosophila* species share the same geographic locations and/or habitat conditions, however, despite the multitude of research on these insects, there is still no consensus for the genetic basis of how the different species remain isolated. The genetic architecture and evolution of mating communication systems represents an evolutionary puzzle as theory suggests that any changes in either signal or receptor would be strongly selected against. A more parsimonious explanation for the diversity in communication systems between species would be one of genetic coupling or pleiotropy. My

thesis suggests that the single *Drosophila* chemosensory gene *Gr8a* can play a role in both signal production and receiver-pheromone and gustatory receptor, adding to the limited body of literature providing exciting empirical evidence for pleiotropy underlying chemical communication.

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Appendix I

Feminization of pheromone-sensing neurons affects mating decisions in *Drosophila* males

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Summary

The response of individual animals to mating signals depends on the sexual identity of the individual and the genetics of the mating targets, which represent the mating social context (social environment). However, how social signals are sensed and integrated during mating decisions remains a mystery. One of the models for understanding mating behaviors in molecular and cellular terms is the male courtship ritual in the fruit fly (*Drosophila melanogaster)*. We have recently shown that a subset of gustatory receptor neurons (GRNs) that are enriched in the male appendages and express the ion channel *ppk23* play a major role in the initiation and maintenance of male courtship via the perception of cuticular contact pheromones, and are likely to represent the main chemosensory pathway that influences mating decisions by males. Here we show that genetic feminization of *ppk23*-expressing GRNs in male flies resulted in a significant increase in male-male sexual attraction without an apparent impact on sexual attraction to females. Furthermore, we show that this increase in male-male sexual attraction is sensory specific, which can be modulated by variable social contexts. Finally, we show that feminization of *ppk23*-expressing sensory neurons lead to major transcriptional shifts, which may explain the altered interpretation of the social environment by feminized males. Together, these data indicate that the sexual cellular identity of pheromone sensing GRNs plays a major role in how individual flies interpret their social environment in the context of mating decisions.

Introduction

Sexually reproducing animals often show sexually dimorphic behaviors. One of the best-characterized models for understanding the role of genetics and neural circuits in controlling sex-specific behaviors is the fruit fly *Drosophila melanogaster* (Anand et al., 2001; Demir and Dickson, 2005; Manoli et al., 2005; Rideout et al., 2010; Ryner et al., 1996; Siwicki and Kravitz, 2009; Villella et al., 1997; Villella and Hall, 2008). Several studies have indicated that sex-specific innate mating behaviors are determined by a dedicated neuronal circuit that is comprised of neurons in the central and peripheral systems, and of which development and function are determined by the sex-specific splicing of the transcription factors *fruitless* (*fru*) and *doublesex* (*dsx*) (Manoli et al., 2005; Rideout et al., 2010; Stockinger et al., 2005).

Cuticular hydrocarbons (CHCs) serve as contact sex pheromones in flies and other insects (Ferveur, 2005; Kent et al., 2008; Krupp et al., 2008; Kuo et al., 2012; Yew et al., 2009). These data suggest that the gustatory system is likely to play an important role in the detection of sex-specific stimuli. This is supported by findings that several members of the gustatory receptor family play a role in the detection of pheromonal signals (Bray and Amrein, 2003; Koganezawa et al., 2010; Miyamoto and Amrein, 2008; Moon et al., 2009; Wang and Anderson, 2010; Wang et al., 2011; Watanabe et al., 2011). In addition, we and others have recently shown that a subset of sexually dimorphic GRNs in the male and female forelegs express both *fru* and the ion channel *ppk23*, and are likely the primary contact pheromone sensory neurons in the adult fly (Lu et al., 2012; Thistle et al., 2012; Toda et al., 2012). Because *ppk23* seems to be exclusively expressed in *fru*-positive gustatory sensory neurons in the male appendages but not in any *fru*-positive central neurons (Lu et al., 2012), studies of the effects of these neurons on male

courtship behavior represent an excellent opportunity to study the relative contribution of the gustatory system to courtship decisions, independent of the brain.

Although stereotypic, both the perception and production of pheromones is highly plastic across sex, species, and physical and social environmental conditions (Billeter et al., 2012; Everaerts et al., 2010; Ferveur, 2005; Kent et al., 2008; Krupp et al., 2008). Here we show that feminization of *ppk23*/*fru*-specific GRNs in the male appendages is sufficient to mimic the effects of mutations in the *fru* locus on male sexual behaviors, independent of the role of fru^{M} in the brain. Our data suggest a simple behavioral model in which *ppk23*-expressing GRNs represent a focal integration point of social environmental cues and the genetic factors that determine cellular sexual identity, which together influence mating decisions of males.

Results

Feminization of ppk23-expressing GRNs induces male-male courtship without altering the innate sexual preference for females

In previous work we have shown that the ion channel *ppk23* and the gustatory neurons that express it play an essential role in the initiation and maintenance of normal male courtship behavior (Lu et al., 2012), by demonstrating that both mutations in *ppk23* and blocking the activity of *ppk23*-expressing GRNs led to a defective male-female courtship behavior. On the other hand, we did not observe any effects of these manipulations on male-male courtship (Lu et al., 2012). We interpreted these data to suggest that *ppk23*-expressing GRNs were mediating the behavioral response of males to aphrodisiac CHCs, which was further confirmed by the reduced behavioral response of *ppk23* mutant males to the excitatory pheromone 7,11-heptacosadiene

(7,11 HD) (Lu et al., 2012; Thistle et al., 2012). However, a calcium imaging study suggested that at least some *ppk23*-expressing GRNs can also respond to the inhibitory pheromone 7-tricosene (7-T) (Thistle et al., 2012). Together, these data suggested that *ppk23*-expressing GRNs represent a heterogeneous population of gustatory-like sensory neurons that are tuned to various classes of contact pheromones.

ppk23-expressing GRNs in the forelegs are sexually dimorphic, and express post-mitotically the sex-determination transcription factor *fruitless (fru)* (Lu et al., 2012; Thistle et al., 2012; Toda et al., 2012). Sex-determination factors such as *fru* and *dsx* are spliced into male or female-specific transcripts by the sex-specific splicing factor *transformer* (*tra*). Previous studies showed that overexpression of the female-specific transcript of *tra* (tra^F) is sufficient to induce female-like differentiation in male tissues, including the nervous system (Ferveur et al., 1997; Ferveur et al., 1995). Consequently, we hypothesized that feminization of *ppk23*-expressing GRNs with ectopic expression of tra^F in otherwise intact males will disrupt their normal function and will lead to similar mating phenotypes we observed in *ppk23* mutant males. To our surprise, males with feminized *ppk23*-expressing GRNs showed robust male-male courtship behaviors measured by male chaining behavior (ANOVA, n=6-8 groups, $p<0.01$, **) (Fig. 1A). However, *ppk23*-feminized males retained their overall sexual preference for courting females when given a choice between wild type male and female targets (Kruskal-Wallis rank sum test, p=0.39) (Fig. 1B), and showed an overall normal courtship behavior towards wild type females measured by courtship latency and index (Figs 1C-d). These observations were in stark contrast to the inhibition of male courtship that we previously observed when *ppk23*-expressing cells were blocked by the ectopic expression of the tetanus toxin in these cells (Lu et al., 2012).

We originally identified *ppk23* as a gustatory-enriched Degenerin/epithelial sodium channel (DEG/ENaC) by screening for genes that were not expressed in the *Poxn* mutant (Lu et al., 2012). Animals that carry mutations in *Poxn* lack all external gustatory sensilla (Awasaki and Kimura, 1997; Boll and Noll, 2002; Dambly-Chaudiere et al., 1992; Nottebohm et al., 1992; Nottebohm et al., 1994; Vervoort et al., 1995). *Poxn* also retains its expression in all postmitotic GRNs and thus serves as an excellent marker for these neurons. As a result, we hypothesized that if the effects of feminizing *ppk23*-expressing GRNs are indeed due to gustatory functions, then feminizing the complete gustatory sensory system in males should lead to a phenotype that is similar to the one we observed in *ppk23*-femizined males. To completely feminize the gustatory system we expressed UAS-*tra*^{F} with a previously published *Poxn*-GAL4 line (Boll and Noll, 2002). As we expected, males with feminized GRNs showed a robust chaining behavior that was indistinguishable from males with the feminization of *ppk23*-expressing GRNs only (Fig. S1A). However, in contrast to *ppk23*-feminized, *Poxn*-feminized males showed a clear preference to courting males over females (Fig. S1B). Nevertheless, when offered a wild type female as a mating target, *Poxn*-feminized males actively courted virgin females with the same tenacity as parental and sibling controls (Fig. S1C-d). These data indicated that courtship decisions in males were also affected by *ppk23*-independent GRNs, and suggested that ectopic feminization of the gustatory sensory system was sufficient to induce a dramatic shift from heterosexual to homosexual behaviors in *Drosophila* males. In both *ppk23*-GAL4 and *Poxn*-GAL4 studies we used the parental lines as wild type controls as has been described in previous studies that used the UAS-*tra*^{F} transgene (Fernandez et al., 2010; Hoxha et al., 2013; Lazareva et al., 2007; Shirangi et al., 2013). Although our data suggest that the homozygous UAS-*tra*^{F} parental line

shows some male chaining behavior, our analyses indicated that chaining is significantly higher when *tra*F was expressed by either *ppk23*-GAL4 or *Poxn*-GAL4. Thus, we conclude that feminization of chemosensory neurons was sufficient to induce chaining behavior in males.

Feminization of ppk23-expressing cells does not increase the sexual attractiveness of manipulated males

Although we did not observe expression of *ppk23* outside the chemosensory system, it is still possible that some of the observed effects on male-male courtship were due to qualitative or quantitative changes in the production of cuticular pheromone signals in feminized males via direct or indirect effects on the pheromone producing oenocytes (Billeter et al., 2009). To test this possibility we first examined the attractiveness of *ppk23*-feminized males as courtship targets for wild type males. We expected that wild type males would become more sexually attracted to feminized males than non-feminized males. However, our data indicated that the attractiveness of manipulated males did not differ from wild type parental controls (Fig. S2A-B). We also analyzed the CHC profiles of feminized and wild type parental males by using gas chromatography (FID) and combined gas chromatography/ mass spectrometry (GC/MS). As with behavior, we did not observe a significant effect of the *ppk23*-feminization on the overall CHC profile or any of the individual compounds (Fig. S2C). These data indicate that the observed increase in male-male courtship in feminized males is due to changes in sensory functions rather than their pheromonal signature.

Feminization of GRNs does not alter gross axonal wiring patterns in the thoracic ganglion

ppk23-expressing GRNs are about two-fold more abundant in male relative to female forelegs,

and show a sexual dimorphic axonal midline crossing in the thoracic ganglia of males but not females (Lu et al., 2012). It has been shown that the axonal midline crossing of GRNs in the male depends on the expression of the male forms of the two main sex-determination transcription factors *fru* and *dsx* (Mellert et al., 2010). Since the splicing of both fru^{M} and dsx^{M} depends on the sex-dependent splicing of *tra* (Robinett et al., 2010; Verhulst et al., 2010), we hypothesized that the ectopic expression of tra^{F} in *ppk23*- or *Poxn*-expressing GRNs in males may have resulted in the inhibition of axonal midline crossing, which subsequently led to aberrant male sexual behaviors. However, anatomical analyses of midline crossing in feminized *ppk23* or *Poxn* males revealed no gross changes in axonal wiring patterns relative to wild type controls (Independent sample t-tests; n=5-6 per genotype) (Fig. 2A-E). We also did not observe any effects of feminization of overall number of *ppk23*-positive cells in males or females (Fig. S3). We cannot explain why feminization by the ectopic expression of tra^{F} did not inhibit axonal midline crossing as was previously reported for direct manipulations of the fru^{M} transcripts in *Poxn* neurons (Mellert et al., 2010). Nevertheless, our data suggest that the behavioral outcomes of chemosensory feminization are not directly related to the status of axonal midline crossing or to the relative abundance of *ppk23*-positive cells in forelegs.

The simplest possible explanation for our findings is that feminization of *ppk23*-expressing GRNs lead to increased male chaining behavior was due to their reduced detection of a inhibitory signals from other males but without affecting their response to excitatory signals from females. To test this hypothesis we examined the behavioral response of males to the inhibitory pheromone 7-T, which is sufficient to inhibit male-male courtship (Billeter et al., 2009; Fernandez et al., 2010; Ferveur and Sureau, 1996; Krupp et al., 2008). Therefore, we

examined the effect of feminization of *ppk23*-expressing GRNs on the behavioral response of manipulated and control males to 7-T. Our data show that in contrast to our hypothesis, feminized males were still sensitive to the inhibitory effects of 7-T when responding to perfumed decoys (Fig. S4A-B). These data suggested that the increase in male-male courtship behavior was not due to a reduced sensing of the principle inhibitory pheromone 7-T, and may suggest that feminized males are actively attracted to other males due to ectopic changes in chemosensory functions.

Our data indicate that males with feminized *ppk23*-expressing cells court conspecific males, but when given a choice between the sexes, still prefer to court conspecifc females. Thus, these data could not resolve whether courting wild types males by *ppk23*-feminized males is an active choice or whether these males will court any possible target in the absence of females. To better distinguish between these two possible explanations we next provided *D. melanogaster* wild type males with heterospecific females from diverse *Drosophila* species of varying phylogenetic distances. Our data indicated that wild type *D. melanogaster* males promiscuously courted most single female targets, independent of phylogenetic distances [ANOVA, n=15-20 for each species except *D.melanogaster* (n=61), *=p<0.05] (Fig. 3A-B). However, females from *D. persimilis*, *D. willistoni* as measured by courtship latency, and *D. willistoni* and *D. mojavensis* as measured by courtship index, were significantly less attractive than other species. As a result, we hypothesized that if *ppk23*-feminized males court other *D. melanogaster* males because they actively find them attractive then when presented with a choice between a *D. melanogaster* male and an unattractive female from a different species then they will still court conspecfic males. Alternatively, if in the absence of *D. melanogaster* female, *ppk23*-femized males will court any

targets without discrimination then they should court both targets equally. To test this we asked *ppk23*-feminized males to choose between between a *D. melanogaster* male and the unattractive *D. willistoni* female. To our surprise, both feminized and wild type control males preferred *D. melanogaster* males to *D. willistoni* females as courtship targets (Kruskal-Wallis rank sum test, n=10-15, p=0.44) (Fig. 3C). These data further supported a model in which male sexual preferences are strongly affected by the available pool of mating targets, and that the decision to court a specific target depends on its relative attractiveness to other possible targets. Furthermore, our data indicate that the feminization of *ppk23*-expressing GRNs leads to an active choice of males as possible targets by shifting how males interpret their social environment when making courtship decisions in complex social environments.

Feminization of ppk23-expressing GRNs leads to changes in the sensory transcriptome in the male appendages

Feminization of *ppk23*-expressing GRNs did not affect the overall cell number in the forelegs of males and females (Fig. S3), or their axonal projection patterns (Fig. 2). Therefore, we hypothesized that an alternative explanation for the observed effects of feminization on male behavior were transcriptional changes in *ppk23*-expressing GRNs. To test this hypothesis we used real-time quantitative RT-PCR to study changes in the expression of fru^F and candidate genes in the male appendages in response to ectopic feminization. We focused our analysis on several genes from the *Gr* and *ppk* families, which have been previously implicated in mediating the gustatory response to contact pheromones (Ben-Shahar et al., 2010; Ben-Shahar et al., 2007; Bray and Amrein, 2003; Lin et al., 2005; Liu et al., 2012; Lu et al., 2012; Miyamoto and Amrein, 2008; Moon et al., 2009; Starostina et al., 2012; Thistle et al., 2012; Toda et al., 2012; Watanabe

et al., 2011), as well genes that encode for feeding related sweet and bitter receptors (*Gr5a* and *Gr66a* respectively) (Dahanukar et al., 2001; Marella et al., 2006; Moon et al., 2006). Although we observed statistically significant changes in the expression levels of several members of the *Gr* and *ppk* families, none of the studied receptor genes showed a dramatic change that may explain the robust behavioral outcome of *ppk23*-feminization (Fig. 4A) (Independent sample t-test; n=4 for each bar; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). Furthermore, although we have previously shown that *ppk23*-expressing GRNs do not overlap anatomically with either sweet (*Gr5a*-expressing GRNs) or bitter (*Gr66a*-expressing GRNs) (Lu et al., 2012), we observed a small but significant increase in *Gr5a* expression in the appendages of feminized males relative to wild type controls (Fig. 4A). Feminized males also showed a significant increase in their sensory sensitivity to sugar (Fig. S5), suggesting that feminization of one GRN type may have indirectly affected the physiology of other feeding related GRNs.

The perception of pheromones by the chemosensory system also depends on rapid enzymatic removal of the perceived chemicals (Feyereisen, 2006; Oakeshott et al., 2010; Wang et al., 2008). In support of this, a gene encoding for a cytochrome P450 enzyme (*Cyp6a20*) was recently implicated in chemosensory functions underlying male-male interactions in *Drosophila* (Wang et al., 2008). Although the exact role of these enzymes in chemosensory biology is not fully understood, it is likely that secreted members of the family play a role in the breakdown of cuticular contact pheromones once they enter the lumen of chemosensory sensillum (Feyereisen, 2006; Willingham and Keil, 2004), where they possibly play a role in the removal or modifications of the sensed pheromones. However, we did not find that *Cyp6a20* was significantly regulated by the feminization of *ppk23*-expressing cells (Fig. 4B) (Independent

sample t-test; n=4 for each bar; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). Nonetheless, several other related family members that cluster in the same genomic region as *Cyp6a20* showed dramatic changes in their expression levels in male appendages in response to feminization, with the most dramatic patterns shown by *Cyp6a17* (Fig. 4B) and *Cyp6d2* (Fig. 4C) (Independent sample t-test; n=4 for each bar; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). Thus, the expression of tra^F in *ppk23*-expressing sensory neurons in males has likely led to major qualitative and quantitative changes in the expression patterns of chemosensory receptors and other genes associated with contact pheromonal signal transduction pathways.

Unexpectedly, we found that the expression of fru^F in the appendages of *ppk23*-feminized males was only about 2-fold higher than in our control line (Fig. 4A). Since our control flies included one copy of the UAS-tra^F transgene, these data suggested that this UAS line might be expressing some levels of tra^F even when GAL4 is not present. To test this, we used PCR to amplify male-specific, female-specific, and common *fru* exons in control and *ppk23*-feminized males, as well as wild type males and females as positive controls. We found that males carrying one copy of the UAS-tra^F transgene expressed fru^M and fru^F (Fig. 4D), indicating partial level of feminization, which is likely due to a "leaky" UAS transgene.

Discussion

Courtship in *Drosophila melanogaster* is one of the best-characterized animal mating behaviors at the molecular and cellular levels (Villella and Hall, 2008). However, we still know relatively little about how flies sense and integrate sex-specific sensory signals (Dickson, 2008). Previous studies of one of the primary sex-determination factors *fru* indicated that mutations in this gene

lead to male chaining behavior (Anand et al., 2001; Demir and Dickson, 2005; Gailey and Hall, 1989; Goodwin et al., 2000; Lee et al., 2000; Manoli et al., 2005; Ryner et al., 1996). In this study we show that genetic feminization of the contact pheromone chemosensory neurons in the male fruit fly appendages is sufficient to phenocopy the classic *fru* behavioral male chaining phenotype (Fig. 1A). However, in contrast to *fru* mutant males who do not discriminate between males and females (Villella et al., 1997), *ppk23*-feminized males still retained their overall preference for courting females over males (Fig. 1B). Thus, our studies indicate that the behavioral impact of feminizing pheromone-sensing neurons on male courtship behavior cannot be explained solely by changes in *fru*-dependent processes. Nevertheless, our data clearly demonstrate that qualitative changes in the expression of chemosensory-related genes are associated with sensory feminization, suggesting that the transcription of some molecular sensory receptors is under the influence of the sex-determination pathway, and may explain some of the differences in pheromone driven behaviors in males and females (Fig. 4).

Previous studies indicated that the decision of a male to court a specific target is mediated by both attractive and repulsive signals (Billeter et al., 2009; Fernandez et al., 2010; Kent et al., 2008; Krupp et al., 2008), and it is the summation of these two opposing forces that determines the length of courtship latency and the intensity of the courtship behavior once a male is committed to a specific target (Ferveur and Sureau, 1996). We found that males with feminized *ppk23*-expressing sensory neurons courted other males, but when given a choice between a male or a female *D. melanogaster* they still preferred to court a female (Fig. 1). These data indicate that feminization did not abolish the ability of these males to discriminate between males and females but rather reduced the inhibition of male-male attraction. A previous study indicated that

wild type males find animals that do not produce any cuticular hydrocarbons, and hence do not have a pheromonal signature, as sexually attractive (Billeter et al., 2009). Thus, the simplest explanation for these data is that feminized males could not sense a male-specific inhibitory pheromone, which resulted in high male-male courtship (Fig. 1). However, feminization of *ppk23*-expresssing neurons did not affect the ability of males to sense excitatory signals present in the female. Thus, when presented with a choice between a male and a female, feminized males still preferred to court females over males. In spite of the simplicity of the above model, further investigations indicated that the increased courtship toward other males by males with feminized *ppk23*-expressing cells was not purely due to the lack of sensing of an inhibitory signal. This is based on data that indicated feminized males still avoided females that were perfumed with 7-T, the main inhibitory cuticular pheromone in *D. melanogaster* (Billeter et al., 2009; Ferveur and Sureau, 1996; Lacaille and Hiroi, 2007; Wang et al., 2011) (Fig. S4). Since the CHC profile of males is typically enriched with 7-T, our data suggest that although feminized males can sense and are repulsed by 7-T, they still find wild type males attractive. These data showed that feminized males were actively attracted to wild type males rather than passively defaulting to males due to the lack of an inhibitory signal, but to a lesser extent relative to their attraction to females. Furthermore, when we gave feminized and wild type males the choice between a *D. melanogaster* male and a *D. willistoni* female, males from all genotypes (including wild type males) preferred to court conspecific males relative to heterospecific females (Fig. 3). Together, these data suggest that males interpret the sensory input into *ppk23*-expresing cells in the context of the social environment they are exposed to. One limitation of our study is the differing strain backgrounds of our transgenic lines, and we cannot exclude that these differences may have an influence on our results. Nevertheless, our data indicate that male sexual decision-making is

strongly influenced by the available mating pool. There is a possibility that the manipulations we employed in our study may have resulted in an intersex phenotype rather than full feminization. However, this would still fit our hypothesis that *ppk23*-expressing cells integrate their own sexual genetic identity with social signals to drive sexual behaviors in males. In addition, the feminization of *ppk23*-expressing neurons can lead to erroneous interpretations of the mating targets pool. These data are in further support of previous studies that showed that the social context of both males and females could affect their courtship behavior as well as the production of pheromones (Billeter et al., 2012; Kent et al., 2008; Krupp et al., 2008).

While our experimental data cannot completely exclude the possibility that feminized males were able to recognize female via non-gustatory pathways, our use of decapitated males and females as targets under red light conditions eliminated vision and the possibility that the courting males recognized sex-specific active behavioral patterns initiated by the courtship targets. Together, these data suggest that changes in the perception of contact pheromones played a role in the abnormal mating behaviors of manipulated males.

Although we have previously shown that *ppk23*-expressing cells do not overlap with sweet sensing (*Gr5a*-expressing) neurons (Lu et al., 2012), males with feminized *ppk23*-expressing neurons showed a small but significant increase in the expression of *Gr5a* receptor in their appendages. Furthermore, feminized males showed higher behavioral sensitivity to sugar stimuli. These data suggest that feminization of pheromone-sensing neurons can affect other classes of gustatory receptor neurons, possibly via indirect mechanisms. These data also further support the possible sensory crosstalk between canonical taste sensory pathways and the pheromonal input

pathways as has been shown for the bitter receptors *Gr66a*, *Gr33a*, and *Gr32a* (Koganezawa et al., 2010; Lacaille et al., 2009; Miyamoto and Amrein, 2008; Moon et al., 2009; Wang et al., 2011).

Previously, we have shown that sexually-dimorphic *ppk23*-expressing neurons represent the primary *fru*-expressing GRNs in the male appendages (Lu et al., 2012). These data suggested that *ppk23*-expressing cells represent the primary subpopulation of contact pheromone-sensing GRNs. In agreement with these data, we found that feminization of all GRNs by using the pan-gustatory driver *Poxn* (Boll and Noll, 2002; Dambly-Chaudiere et al., 1992) also led to male chaining behavior (Fig. S1). However, in stark contrast to male-male courtship behaviors of *fru* mutant males (Gailey and Hall, 1989; Villella et al., 1997) and in males with feminized *ppk23*-expressing cells, males with a feminized gustatory system preferred males to females (Fig. S1). Since *Poxn*-GAL4 is expressed in all gustatory receptor neurons including *fru*-expressing neurons in the proboscis, these data suggest that additional gustatory neurons that do not express *ppk23* are also likely to play a role in the sexual decision making process of male *Drosophila*.

Although we have previously shown that contact pheromone sensory neurons are sexually dimorphic in terms of their axonal projection patterns (Lu et al., 2012), feminization of gustatory receptor cells affected the behavior of males without an obvious gross impact on male-specific axonal patterns (Fig. 2). This outcome was surprising since previously published studies showed that manipulation of the *fru*-dependent sex determination pathway had a significant effect on axonal midline crossing of gustatory neurons in males and females (Mellert et al., 2010; Possidente and Murphey, 1989). It is possible that the lack of effect of tra^{F} with the *ppk23-GAL4*

driver is due to the late onset of *ppk23* transcription during development. *ppk23* expression begins in the late pupal stages (Fig. S6A), and therefore *ppk23-GAL4* may not affect midline crossing in the nervous system. Rather, *ppk23* may act in the maintenance of sex-specific circuits post-developmentally. *Poxn* expression, however, begins in the embryonic stage (Fig. S6B) and so it remains unclear why the expression of tra^F with the *poxn* driver did not alter neuronal wiring patterns. Consequently, based on the current understanding of the sex-determination pathway in *Drosophila*, we expected that ectopic expression of tra^F in males would phenocopy what was reported in previous studies since tra^F signaling is upstream from *fru*. Furthermore, *ppk23*-feminized males did show a significant increase in the fru^F specific transcripts in their appendages (Fig. 4A). One possible genetic explanation to the discrepancy in our findings is that we ectopically expressed tra^F in the background of wild type *tra* locus. Therefore, it is possible that the endogenous male-specific sex-determination genetic cascade was sufficient to maintain the male-specific axonal projection pattern. Nevertheless, our data strongly support the hypothesis that certain aspects of the sexual dimorphism observed in the *ppk23*-expressing cells do not depend on their abundance in males versus females or their sexually dimorphic axonal midline crossing.

The studies we report here contribute to a better understanding of the role of the sex-determination pathway in regulating the sensory inputs used by males to make mating related decisions. Our data support a model in which *ppk23* pheromone sensing neurons represent a focal element in the sex circuit, which determines how males respond to their social environment to achieve adaptive mating decisions. Our approach indicates that by taking advantage of mosaic males in which only one class of sensory neurons is female-like in otherwise intact males would

enable us to start dissecting in high detail the genetic networks that determine sexual decision making in males and females, independent of higher central neuronal functions.

Materials and Methods

Fruit fly strains and genetics. All fly stocks were maintained on standard cornmeal medium at 25 ºC under 12:12 light-dark cycle. The *ppk23 promoter-GAL4* line was described previously (Lu et al., 2012). *UAS-tra*^{F} flies were from Ralph Greenspan. Unless mentioned, all other fly strains used in our studies were obtained from the Bloomington Stock Center. Non-*D. melanogaster* fruit fly species were obtained from the San Diego Species Stock Center. Specific lines used were: *D. simulans* 14011-0251.192, *D. sechellia* 14021-0248.03, *D. yakuba* 14021-0261.01, *D. erecta* 14021-0224.00, *D. ananassae* 14024-0371.16, *D. pseudoobscura* 14011-0121.104, *D. persimilis* 14011-0111.50, *D. willistoni* 14030-0811.35, *D. mojavensis* 15081-1352.23, and *D. virilis* 15010-1051.118. The used species were chosen based on whole genome availability as well as coverage of the major groups across the *Drosophila* lineage. All species were maintained on standard cornmeal medium except for *D. mojavensis*, *D. persimilis* and *D. pseudoobscura*, which were supplemented with banana, and D. *sechellia*, which was supplemented with noni fruit leather (*Morinda citrifolia*).

Real-time Quantitative RT-PCR assays. qRT-PCR was assayed as previously described (Lu et al., 2012). Briefly, fly appendages were separated by repeated vortexing of whole flies frozen in liquid nitrogen. Total RNA extraction and cDNA synthesis were performed by using Trizol and SuperScript II reverse transcriptase respectively (Invitrogen). qPCR assays were performed on an ABI7500 machine with ABI SYBRGreen chemistry. The housekeeping gene *rp49* was used as an RNA loading control. Ct data were transformed according to the ΔΔCt method and are

represented as relative values (Ben-Shahar et al., 2002). See supplementary table for gene-specific primers used in our study.

RT-PCR assays. Total RNA extraction and cDNA synthesis from fly appendages were performed as described above. To identify the presence of *fru* transcripts in our samples we conducted a PCR-based screen using the following forward primers: male specific fru^M (GGCGACGTCACAGGATTATT), female specific fru^F (TCAATCAACACTCAACCCGA), common fru^C (TGGAACAATCATCCCACAAA), and a common fru^R reverse primer (AGTCGGAGCGGTAGTTCAGA). PCR's were performed with Taq supermix (Lamda) in 25 uL reactions, and then separated on a 1.0% agarose gel (Fig. 4D).

Chemical Analysis of Cuticular Hydrocarbons (CHC). Male flies that were 4-7 days old were kept frozen in -80°C until extraction. Parental genotypes *ppk23-GAL4* and *UAS-*tra^F were used as controls. For CHC extraction, groups of 5 frozen flies were shaken in a glass vial with 200 µL of Hexane. 100ng n-octadecane was added to the extracts (C-18), as an internal standard. Samples from the extract were analyzed using gas chromatography (CP 3900; Varian). Quantitative analyses of CHCs were done with a DB-1 fused silica column that was temperature-programmed from 150°C (1 min of initial hold) at 5°C/min to 300°C. Compound quantification was done by peak integration in comparison with the internal standard. Peaks identity was verified by using a 5975 Supersonic Molecular Beam (SMB) GC-MS with cold EI (Amirav et al., 2008) (Aviv Analytical model 5975-SMB, www.avivanalytical.com), which provides an unambiguous molecular ion as well as pronounced ion fragments at the branching points of branched hydrocarbons. The identity of the compounds in the extracts were in agreement with previously published data (Everaerts et al., 2010).

Histochemistry and microscopy. Immunostainings of thoracic ganglia was done as previously described (Lu et al., 2012). In short, freshly dissected brain and thoracic ganglia from flies that express a membrane tethered version of EGFP (CD8::GFP) in either *ppk23* or *Poxn* expressing neurons were fixed in 4% paraformaldehyde and washed in PBT. The specimens were co-stained with anti-GFP (Invitrogen) and the neuropil marker anti-nc82 (Developmental Studies Hybridoma Bank, University of Iowa) and mounted on slides with Slowfade Gold antifade reagent (Invitrogen) according to well-established protocols (Wu and Luo, 2006). All images were taken with a Nikon A1 confocal microscope. Shown images were constructed from optical Z-stacks and analyzed using the Nikon NIS-Elements software package.

Courtship behavior. Courtship was assayed with four to seven day old males as previously described (Ben-Shahar et al., 2010; Lu et al., 2012). In short, courtship assays were done under red light conditions unless differently stated and targets were decapitated. Courtship latency was calculated as the time from female introduction until the male showed obvious courtship behavior such as orientation coupled with wing extensions. Once courtship began, courtship index was calculated as the proportion of time a male spent in any courtship-related activity during a 10min period or until mating occurred. For the 7-T treatment, groups of CO_2 anesthetized virgin 4-5 days old females were placed in small glass vials that were coated with a thin layer of the compound. Females were then perfumed by three repeats of 20s gentle vortexing followed by a 20s rest interval according to previously published protocols (Billeter et al., 2009). The 7-T courtship assays were performed under white light in a circular courtship arena (22mm in diameter).

Interspecific single-pair tests. *D. melanogaster* virgin males were collected upon eclosion and kept separately in small vials (12 x 75mm). Female virgin flies of all species were collected upon eclosion and kept in groups of up to 10 flies from a single-species. All vials contained standard cornmeal medium. Flies were aged 4-7 days under constant conditions of 25 °C and a 12:12 light-dark cycle before behavioral experiments to ensure reproductive maturation. Interspecific no-choice tests were then carried out in behavioral chambers as previously described (Lu et al., 2012).

Chaining behavior. Male chaining was assayed with eight male in a 22mm diameter circular

arena as previously described (Lu et al., 2012). Chaining index was calculated as the proportion of time in which at least three males showed chaining courtship to each other during a 10min observation.

Choice behavior. Choice was assayed by introducing a single focal male and two decapitated targets. Flies were videotaped and analyzed for the duration of time the focal male spent courting each of the two targets. The courtship choice index was calculated [(duration of courtship of target A – duration of courtship of target B)/total courtship time]. Courtship time was measured from the moment the male started courting one of the targets. Total assay time was kept at 10min.

Proboscis extension reflex. (PER) assays were as previously described (Lu et al., 2012). In short, 1-day old flies were starved for approximately 24 hours, then immobilized by chilling on ice and mounted ventral-side-up using myristic acid. Flies were allowed to recover for two hours under humid conditions. Flies were satiated with water prior to the PER training. Flies were tested by introducing a drop of the test solution to a foreleg. Only full PER responses were recorded as positive. Each fly was exposed three times to the same stimulus in each concentration with water application between each trial. 'Responders' were classified as such if they responded to at least 2 out of 3 trials. The responding index represents the sum of all positive responses of an individual animal to a specific sequence of tarsal stimuli.

Statistical Procedures. All statistical tests were performed using the R statistical package. Data were tested for normality by using the Shapiro-Wilk test. Two-sample t-tests and one-way ANOVA tests were used for parametric statistics and the two-samples Wilcoxon test and

Kruskal-Wallis rank sum test were used for non-parametric tests. Chi-square tests were used for frequency-based data.

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Competing interests

The authors have no competing interests to declare.

Author Contributions

B.L. and Y.B. designed experiments. B.L., K.M.Z, R.S., and A.H. executed experiments and analyzed data. B.L., K.M.Z., A.H. and Y.B. wrote the paper.

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Figures

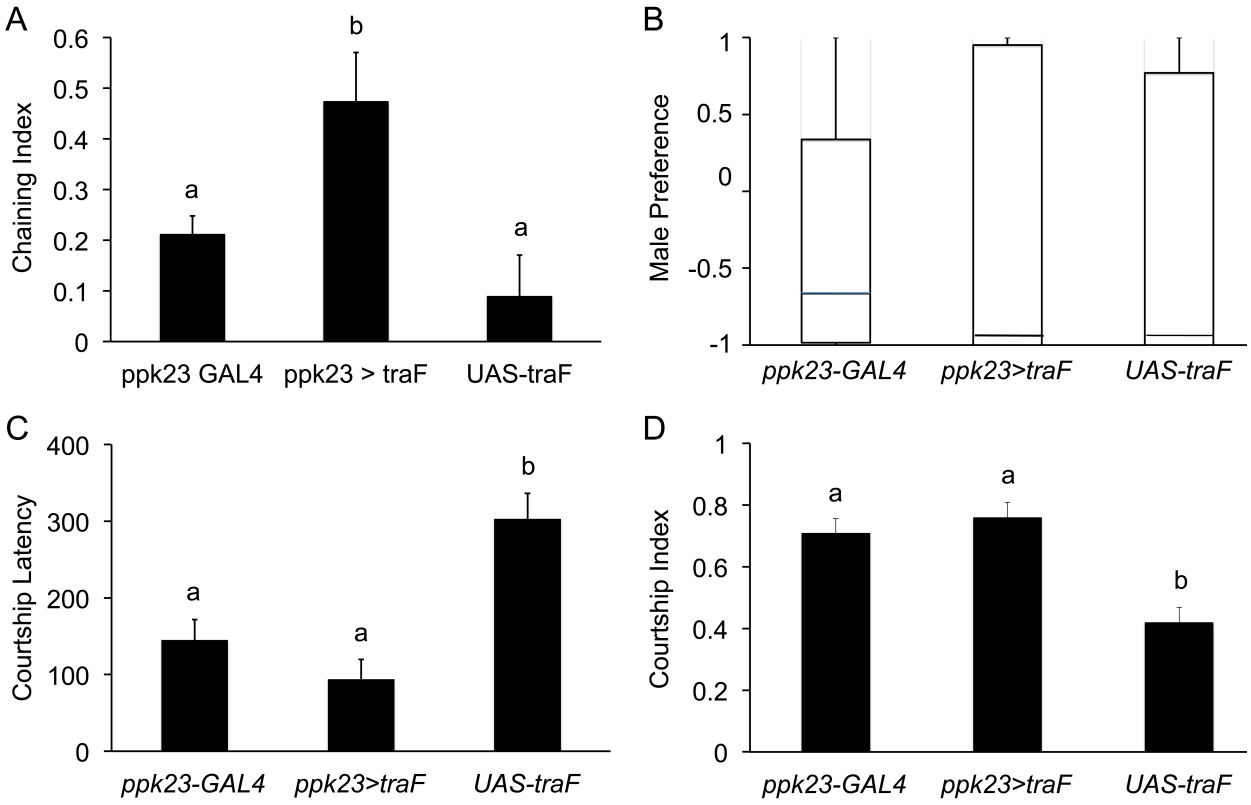

Figure 1: Males with feminized *ppk23*-expressing GRNs show increased male-male courtship behavior. (*A*) Male-male chaining index in feminized flies (*ppk23*>*tra*^{F}) and two parental controls (*ppk23*-GAL4, UAS-*tra*^{F}). Feminized males showed higher male chaining behavior relative to males from parental lines. (*B*) Feminized males preferred females to males in choice courtship assays. Boxplots show the distribution of choice behaviors (1, male; -1 female). (*C-D*) Feminized males show normal courtship behavior towards wild type females. Feminization of *ppk23*-expressing GRNs had no effect on either latency or courtship index relative to parental *ppk23*-GAL4 males. UAS-*tra*^{F} parental males showed consistent longer latency (*C*) and reduced courtship index (*D*), which were likely due to unrelated factors present in the genetic background of this specific transgenic line. The different letters (a,b) in parts A, C, and D represent groups that are significantly different from each other based on ANOVA *post hoc* tests.

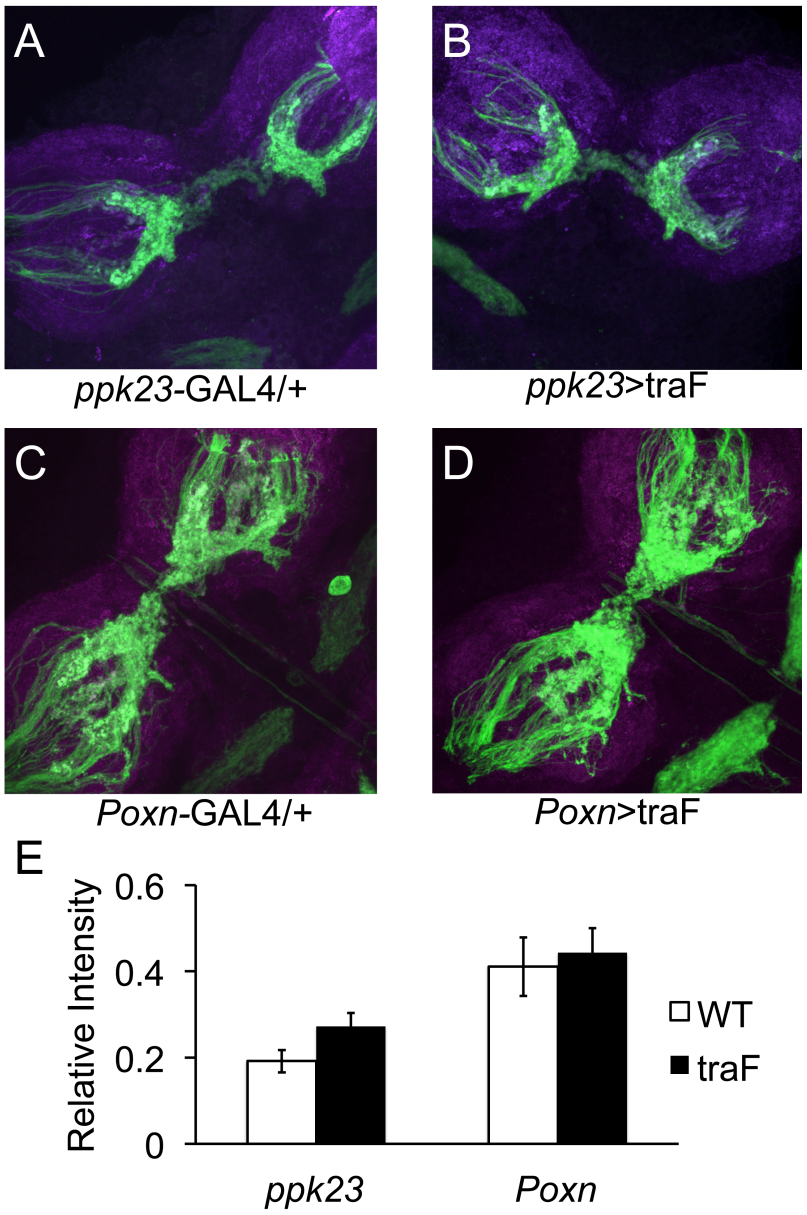

Figure 2. Feminization of *ppk23*-expressing chemosensory neurons does not affect their gross axonal projection patterns. (*A*) Membrane-tethered GFP (UAS-mCD8::GFP) was expressed by *ppk23*-GAL4 (wild type pattern). (*B*) UAS-mCD8::GFP was co-expressed with UAS-*tra*^{F} by *ppk23*-GAL4. (*C*) mCD8::GFP was expressed by the pan-gustatory *Poxn*-GAL4 line. (*D*) UAS-mCD8::GFP was co-expressed with UAS-*tra*^{F} by *Poxn*-GAL4. (*E*) Quantification of relative fluorescence intensity in the midline-crossing region. No significant differences were found between control and *tra*^{F}-expressing males with either GAL4 lines.

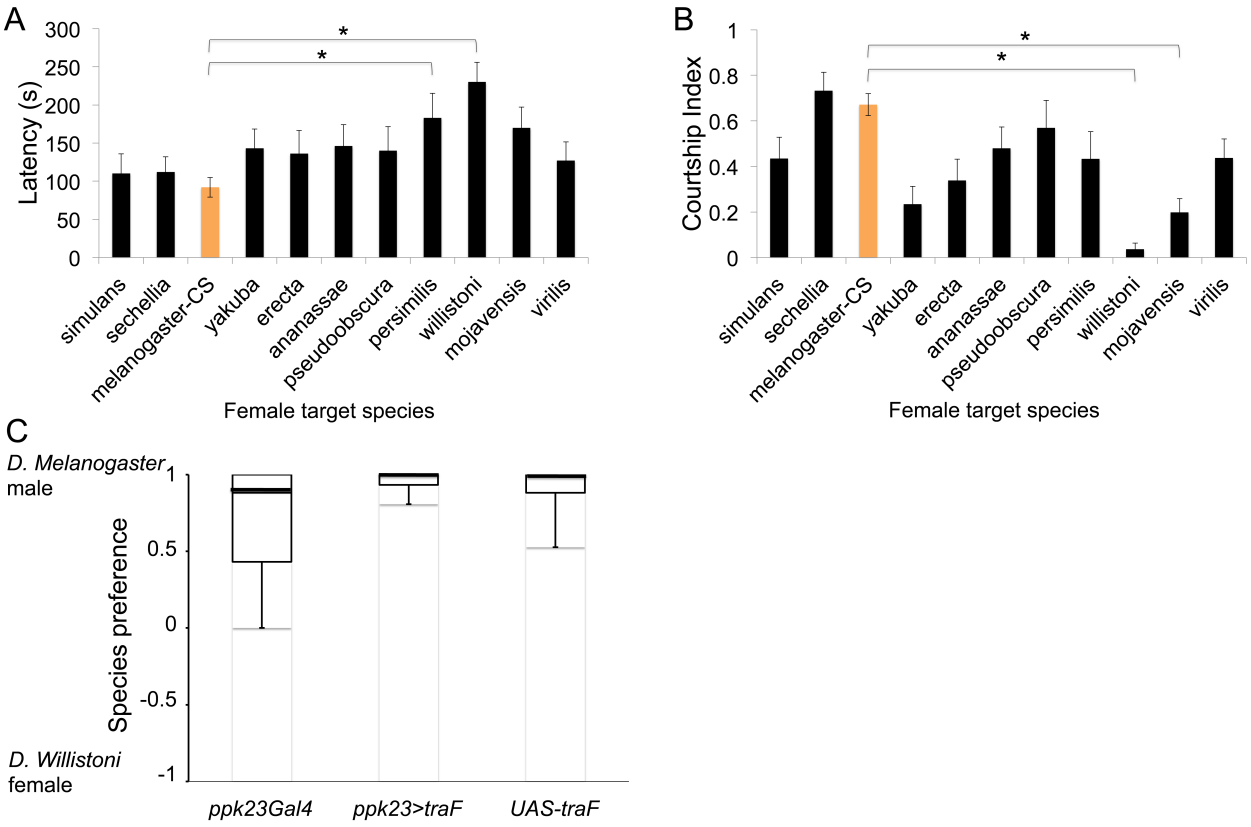

Figure 3. *D. melanogaster* males prefer to court conspecific males over females of a distant species. Wild type *D. melanogaster* males courted females from other species with varying degrees of intensity as measured by the courtship latency (*A*) and the courtship index (*B*) . (*C*) In choice assays, *D. melanogaster* males of all tested genotypes chose to court conspecific males over females of *D. willistoni*. Boxplots represent the distribution of male mating choices. No significant differences were found between feminized flies and the parental controls.

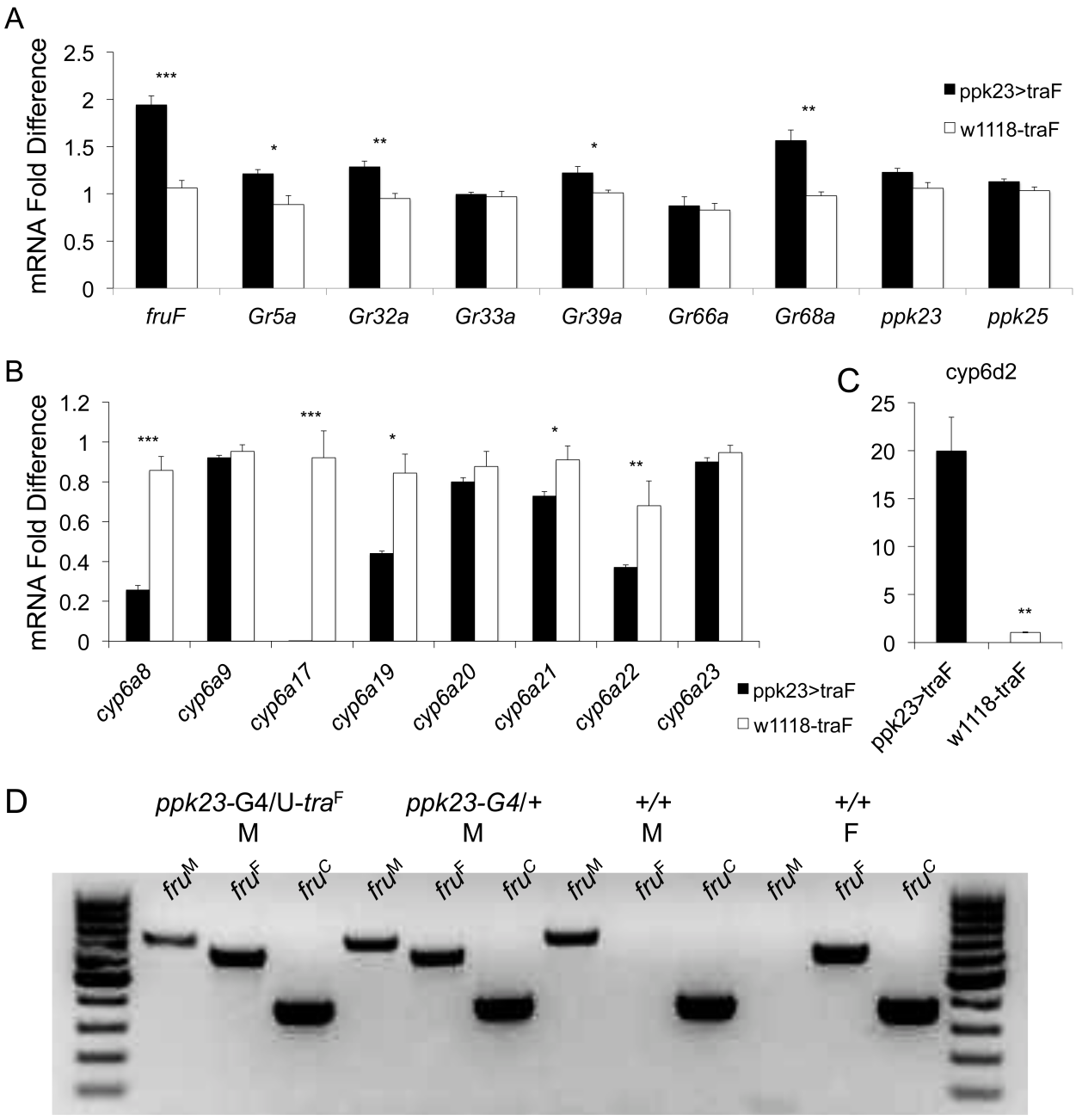

Figure 4. Feminization of *ppk23*-expressing cells leads to significant shifts in the chemosensory transcriptome in male appendages. (*A*) Real-time quantitative RT-PCR analyses of chemosensory genes that have been previously implicated in pheromonal sensing. Analyses were of total RNA extracted from male appendages from feminized flies (w^{1118};*ppk23-GAL4*/UAS-tra^{F}) and wild type controls (w^{1118}/UAS-tra^{F}). (*B*) Real-time quantitative RT-PCR analyses of members of the Cytochrome P450 family, subfamily 6. The expression of *Cyp6d2* is shown separately since this gene was regulated in the opposite direction relative to all other *Cyp6* genes (*C*). (*D*) PCR analyses of sex-specific *fru* transcripts in appendages. fru^{M}, male-specific; fru^{F}, female-specific; fru^{C}, common exons. M=male, F=female, +/+=w^{1118}.

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Appendix II

The Genetic Architecture of Degenerin/Epithelial Sodium Channels in *Drosophila*

Research article

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Key words: Degenerin/epithelial sodium channel, Chemosensation, Mechanosensation, Phylogeny, fruit fly

Running title: *ppk* genes in *Drosophila*

Abbreviations: DEG/ENaC: Degenerin/ epithelial sodium channel, PDB: Protein Data Bank, HMM: Hidden Markov Model, *ppk*: *pickpocket*, ASIC: Acid-Sensing Ion Channel, rpk: rippedpocket

Abstract

Degenerin/epithelial sodium channels (DEG/ENaC) represent a large family of animal-specific membrane proteins. Although the physiological functions of most family members are not known, some have been shown to act as non-voltage gated, amiloride-sensitive sodium channels. The DEG/ENaC family is exceptionally large in genomes of *Drosophila* species relative to vertebrates and other insects. To elucidate the evolutionary history of the DEG/ENaC family in *Drosophila*, we took advantage of the genomic and genetic information available for 12 *Drosophila* species that represent all the major species groups in the *Drosophila* clade. We have identified 31 family members (termed *pickpocket* genes) in *Drosophila melanogaster*, which can be divided into six subfamilies, which are represented in all 12 species. Structure prediction analyses suggested that some subunits evolved unique structural features in the large extracellular domain, possibly supporting mechanosensory functions. This is further supported by experimental data that show that both *ppk1* and *ppk26* are expressed in multidendritic neurons, which can sense mechanical nociceptive stimuli in larvae. We also identified representative genes from five out of the six DEG/ENaC subfamilies in a mosquito genome, suggesting that the core DEG/ENaC subfamilies were already present early in the dipteran radiation. Spatial and temporal analyses of expression patterns of the various *pickpocket* genes indicated that paralogous genes often show very different expression patterns, possibly indicating that gene duplication events have led to new physiological or cellular functions rather than redundancy. In summary, our analyses support a rapid early diversification of the DEG/ENaC family in Diptera followed by physiological and/or cellular specialization. Some members of the family may have diversified to support the physiological functions of a yet unknown class of ligands.

Introduction

All cells use a complex array of ion channels to maintain the appropriate ionic gradients across membrane barriers, including the plasma membrane and intracellular compartments and organelles. One enigmatic group of ion channels is the Degenerin/epithelial Na^+ channel (DEG/ENaC) family. Although the physiological functions of most family members are not well understood, at least some members seem to act as non-voltage gated, amiloride-sensitive sodium channels (Bianchi and Driscoll, 2002; Garty and Palmer, 1997). Various natural ligands and mechanical stimuli can activate or modulate channel functions. These include the neuropeptides FMRFamide (Askwith *et al.*, 2000; Durrnagel *et al.*, 2010; Golubovic *et al.*, 2007; Green *et al.*, 1994; Kellenberger and Schild, 2002; Lingueglia *et al.*, 1995; Xie *et al.*, 2003), FFamide, SFamide (Deval *et al.*, 2003; Sherwood and Askwith, 2008, 2009), and dynorphin-related opioid peptides (Sherwood and Askwith, 2009). In addition, some mammalian family members are gated by extracellular protons (Benson *et al.*, 2002; Price *et al.*, 2001; Waldmann *et al.*, 1997; Xie *et al.*, 2003; Xiong *et al.*, 2004). Recently, several sulfhydryl compounds (Cho and Askwith, 2007) and small polyamines such as agmatine (Yu *et al.*, 2010) were also shown to modulate the channel functions of specific mammalian family members. Finally, data also support a role for specific DEG/ENaC subunits in pheromone-dependent behaviors as well as in chemosensory functions underlying male courtship behaviors in *Drosophila* (Ben-Shahar, 2011; Ben-Shahar *et al.*, 2010; Ben-Shahar *et al.*, 2007; Lin *et al.*, 2005; Lu *et al.*, 2012; Starostina *et al.*, 2012; Thistle *et al.*, 2012; Toda *et al.*).

DEG/ENaC family members have also been implicated in mechanosensation in *Caenorhabditis elegans*, mammals, and *Drosophila* (Arnadottir *et al.*, 2011; Bazopoulou *et al.*, 2007; Geffeney

et al., 2011; Lu *et al.*, 2009; O'Hagan *et al.*, 2005; Price *et al.*, 2001; Simon *et al.*, 2010; Tsubouchi *et al.*, 2012; Zhang *et al.*, 2004; Zhong *et al.*, 2010). Together, these data indicate that DEG/ENaC channels have evolved to serve many different physiological functions, acting as ionotropic receptors to diverse extracellular stimuli.

Functional and structural studies of DEG/ENaC channels demonstrated that channels are likely hetero or homotrimeric (Benson *et al.*, 2002; Canessa *et al.*, 1994; Eskandari *et al.*, 1999; Jasti *et al.*, 2007; Kellenberger and Schild, 2002; Zha *et al.*, 2009b). Electrophysiological studies indicated that subunit composition has a significant effect on the pharmacological and kinetic properties of assembled channels, suggesting that channel subunit composition plays a critical regulatory mechanism (Askwith *et al.*, 2004; Benson *et al.*, 2002; Chu *et al.*, 2004; Xie *et al.*, 2003; Zha *et al.*, 2009a; Zhang *et al.*, 2008). Hence, channel subunit diversity in a single animal is likely to represent diversity in activating stimuli and/or complex channel regulation.

Although the DEG/ENaC family is highly diverse across animalia, all family members share several highly conserved structural and topological features (Bianchi, 2007; Bianchi and Driscoll, 2002; Corey and Garcia-Anoveros, 1996; Tavernarakis and Driscoll, 2000, 2001). Conserved topologies include two transmembrane helixes, two short intracellular domains, and a large cysteine-rich extracellular loop (Ben-Shahar, 2011) (Fig. 1).

Surprisingly, mammalian genomes encode only eight to nine independent DEG/ENaC subunits, while the genomes of the worm *C. elegans* and various *Drosophila* species harbor a significantly larger number of DEG/ENaC-like genes (31 in *D. melanogaster* and 30 in *C. elegans*)

(Bazopoulou *et al.*, 2007; Ben-Shahar, 2011; Liu *et al.*, 2003a; Liu *et al.*, 2003b; Studer *et al.*, 2011). Consequently, DEG/ENaC genes represent one of the largest ion channel families in the *Drosophila* genome. The high diversification of DEG/ENaC protein sequences across distant animal species makes it difficult to evaluate whether the family expanded in some invertebrate species or whether it contracted in vertebrates. Nevertheless, the remarkable diversity of *ppk* genes in *Drosophila* suggests two alternative hypotheses. The first would suggest DEG/ENaC ion channels serve a wider range of physiological functions relative to their roles in mammals. An alternative hypothesis would be that DEG/ENaC channels in *Drosophila* evolved to serve highly specialized functions, predicting that each specific DEG/ENaC channel type in flies is responsible for a narrow slice of the physiological functions performed by a mammalian family member. However, identifying physiological and functional homology between family members across distant species is often impossible due to the poor overall protein sequence conservation of the extracellular loop domains. Thus, protein alignment analyses alone are typically not sufficient to draw physiological homology conclusions. Consequently, newly identified family members typically require physiological analyses *de novo*.

The rising interest in DEG/ENaC-dependent signaling, their emerging importance in diverse physiological functions, and their high variability across different animal genomes suggests these ion channels may have played an important role in animal evolution. Here we reason that the dramatic diversity of the DEG/ENaC family in the *Drosophila* lineage represents an excellent opportunity to use evolutionary and molecular studies to gain new insights into the possible unique role of these channels in diverse physiological systems in general and insect biology in particular.

Materials and Methods

Phylogenetic analyses

Drosophila melanogaster ppk family member protein sequences were mined in FlyBase and multiply aligned using Clustal Omega (Sievers *et al.*, 2011). To determine the best model of protein evolution for our data, we entered the alignment into ProtTest v 2.4. The appropriate substitution matrix was selected from the Akaike Information Criterion and Bayesian Information Criterion scores (Abascal *et al.*, 2005; Darriba *et al.*, 2011; Drummond and Strimmer, 2001; Guindon and Gascuel, 2003). Phylogenetic analysis was then completed using a maximum likelihood approach and rapid bootstrapping algorithm within RAxML v 7.2.8 Black Box (Stamatakis, 2006; Stamatakis *et al.*, 2008), on the Cipres web portal (Miller *et al.*, 2010). Visualizations of the bipartition files were made using FigTree v 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

Expression of ppk genes

Expression patterns of each member of the *ppk* gene family across different fly tissues were mined from FlyAtlas (Chintapalli *et al.*, 2007). Microarray expression data from four independent microarrays were normalized and then graphed according to the expression level in different tissues. Temporal expression patterns of the *ppk* gene family were extracted from the modENCODE RNA-sequencing database (Celniker *et al.*, 2009; Graveley *et al.*, 2011). Normalized maximum expression was represented at different developmental stages, from the embryo to the adult fly in both males and females. To observe the spatial expression patterns of *ppk* and *ppk26* at a single cell resolution, we used the UAS-GAL4 binary expression system (Brand and Perrimon, 1993) to express a membrane tethered version of EGFP (UAS-

mCD8::GFP) using a previously published *ppk*-GAL4 line and a new *ppk26*-GAL4 line we have generated. *ppk*-GAL4 line was obtained from the Bloomington Drosophila Stock Center (stock# 32078). The *ppk26*-GAL4 line was produced by amplifying a 2.2Kb fragment that included the first intron as well as sequences upstream of *ppk26* transcriptional start site (coordinates were 3L: 7447230-7449432 in release 5.47 of the *Drosophila* genome)

PPK protein structure modeling

There are currently seven different accession numbers for structural models of DEG/ENaC channels in the PDB database, all which are based on the chicken ASIC1a protein. We chose to base our structural analyses of the *Drosophila ppk* gene family on the original 2QTS model (Jasti *et al.*, 2007) because of the following reasons: 1) The 2QTS model has the best resolution (1.9Å), which serves better as a template of homology modeling; 2) 2QTS is a ligand-free model, which we predicted would work better as a modeling template since ASIC1a is a proton receptor, which is not necessarily a general property of DEG/ENaC channels. To generate structural predictions *in silico*, all PPK reference sequences and the template sequence (PDB ID: 2QTS) were aligned onto Hidden Markov Model (HMM) of amiloride-sensitive sodium channel (ASC) family from PFAM (Punta *et al.*, 2012) (PFAM ID: PF00858) by the program *hmmalign* in HMMER3 (Finn *et al.*, 2011) and visualized by CLC Sequence Viewer. From the pair-wise sequence alignment of each PPK protein and the template, multiple structural models were generated by MODELLER with default homology modeling protocol (Sali and Blundell, 1993). The model with the best score was selected for further analysis. The molecular graphics software UCSF Chimera was used for structural visualization and analysis (Pettersen *et al.*, 2004).

Results and Discussion

The *ppk* family in *Drosophila melanogaster*

Previous studies have identified several DEG/ENaC family members, which were termed *pickpocket* (*ppk*) genes (Darboux *et al.*, 1998a; Liu *et al.*, 2003a; Liu *et al.*, 2003b). However, a comprehensive scan of the fly genome for all family members has not been performed to date. We used a combination of current genome annotations as well as various homology search engines to identify 31 independent genes encoding for family members, which we named *ppk-ppk31* in complete agreement with prior annotations (Table 1).

Alignment of all identified PPK sequences revealed a highly conserved cysteine-enriched domain, which contains 5 disulphide bonds by ten highly conserved cysteines in the thumb domain (Fig. 1A-1B). Unrooted protein phylogenetic analysis of all identified *ppk* genes in the *D. melanogaster* genome indicated that this protein family is comprised of at least six distinct subfamilies (labeled as I-VI, Fig. 2). Overall, the relationship between *ppk* genes in subfamilies III, IV, and V are well resolved and supported by high bootstrap values. However, few genes such as *ppk17* and *ppk23* are not well resolved in our phylogeny, despite multiple (N=4) runs of the alignment and phylogenetic tree programs, which produced the same results for each run. The inability to resolve certain *ppk* relationships is likely due to the high amount of divergence in amino acid sequence between *ppk* family members (Table S1).

ppk genes are highly conserved in the *Drosophila* lineage

We subsequently extended our gene search analyses to the sequenced genomes of additional 11 *Drosophila* species as well as to the genome of *Anopheles gambiae* (African malaria mosquito),

which served as a dipteran outgroup (Table S2) (Holt *et al.*, 2002). These analyses revealed that the majority of the *D. melanogaster ppk* radiation is preserved in all 12 sequenced *Drosophila* genomes (Bhutkar *et al.*, 2008; Singh *et al.*, 2009), indicating *ppk* diversification occurred early in the evolution of the *Drosophila* lineage.

Expression patterns, structural variations, and predictions of function

Analyses of mRNA expression levels across various *D. melanogaster* tissues (Fig. 3A) and developmental stages (Fig. 3B) indicated that individual *ppk* family members show different expression profiles in both mRNA expression level and temporal and spatial expression patterns. These data suggest that this family has evolved to serve a wide variety of physiological functions. Though a handful of subunits have been implicated in mechanosensation and chemosensory perception, the contribution of sequence variation to physiological function remains unclear. Of particular interest is subfamily V, which includes the *ppk*, *rpk*, and *ppk26* cluster (Fig. 2, 4). Both *rpk* and *ppk* have been implicated in mechanosensation in larvae, though in different types of multidendritic neurons, and are likely to have similar but independent functions in neurons (Adams *et al.*, 1998; Kim *et al.*, 2012; Tsubouchi *et al.*, 2012; Zhong *et al.*, 2010). The spatial expression pattern of *ppk26*, which is a close paralogue of the *ppk* and *rpk* subunits is very similar to *ppk* suggesting the two subunits might be co-expressed (Fig.3A). To further explore this, we generated a transgenic *Drosophila* line that can report the expression patterns of the gene using the UAS-GAL4 system (Brand and Perrimon, 1993). As predicted by the mRNA expression data, the expression of the *ppk26* gene is enriched in class IV multidendritic sensory neurons, which also express *ppk* (Fig 4). These data suggest that *ppk26* and *ppk* are either redundant or are co-required for some aspect of mechanosensation in these nociceptive neurons.

In sum, though the functions of all DEG/ENaC subunits are not yet known, we hypothesize that *ppk*, *rpk*, and *ppk26* , which show sequence and structural similarities and are expressed in multidendritic neurons, may have similar functions in nociceptive mechanosensation.

Subfamily III is not present in mosquitoes.

As expected, *ppk* family gene conservation between the *D. melanogaster* and the mosquito genomes was lower than across the *Drosophila* lineage (Table S2). We identified only 18 family members in the genome of *A. gambiae* of which 17 had homologs in the *Drosophila* genome and one that seemed to be a mosquito-specific subunit (AGAP006704, Table S2). These data suggest that the extreme diversity we observed in the *Drosophila* lineage is not shared by all dipteran species.

Closer examination of the conservation of *Drosophila ppk* subfamilies in *A. gambiae* revealed that none of the genes represented in subfamily III were present in the mosquito genome, suggesting this subfamily is not common in all dipteran species. (Fig. 2 and Table S2). In contrast, we have indentified at least one homologous gene from each of the remaining *ppk* subfamilies in the mosquito genome (Table S2). These data may suggest that each *ppk* subfamily (with the exception subfamily III) represents a core DEG/ENaC physiological function in Diptera.

Diversity, duplications, gene syntenies, and sequence homologies

Examination of overall gene conservation across all sequenced *Drosophila* species indicated that protein phylogeny followed closely the predicted species phylogeny (Clark *et al.*, 2007). We

examined in more detail several subfamilies of conserved *ppk* genes across the 12 sequenced *Drosophila* genomes as well as the malaria mosquito *A. gambiae*. We first examined the highly conserved subgroup that included *ppk*, *rpk*, and *ppk26*. All three genes are highly conserved across all 12 genomes (Table S2).

While each *Drosophila* genome includes one subunit that corresponds most closely to *ppk*, *rpk*, or *ppk26*, the mosquito genome encodes four related subunits, all of which are clustered with the *Drosophila ppk26* (Table S2). These data suggest that *ppk26* represents an early dipteran subunit, which may have independently diversified in the *Drosophila* and mosquito lineages.

Nine of the 31 *ppk* genes we have identified in the *D. melanogaster* genome are chromosomally clustered (Fig. 5). Protein phylogeny indicated that the majority of genomic clusters were likely the result of gene duplications since the clustered genes showed high sequence similarities and belonged to the same *ppk* subfamilies (Boxed genes names in Fig. 2). An exception is *ppk18*, which is clustered with *ppk11* and *ppk16* (Fig. 5B), two less related subunits (Fig. 2). These data suggest that the clustering of these three subunits might have been the result of selection underlying shared physiological and/or cellular functions. *ppk11* has been implicated in salt taste (Liu *et al.*, 2003b). We speculate that these three subunits might contribute to salt taste in *Drosophila* by forming the sodium sensitive ion channel. (Adams *et al.*, 1997; Chandrashekar *et al.*, 2006; Chandrashekar *et al.*, 2010; McDonald *et al.*, 1995; Snyder *et al.*, 1995). We found that all identified *D. melanogaster ppk* genomic clusters are conserved across all 12 *Drosophila* species genomes (not shown), indicating that the molecular events that led to clusters formation happened early in the species radiation of the *Drosophila* genus.

In addition to linear protein sequence analyses, we also built structural models of all PPK proteins by using the published crystal structure of the chicken Acid-Sensing Ion Channel (Jasti *et al.*, 2007) as a guide. According to the protein conservation information from multiple alignment of the *ppk* family, we rendered a general *Drosophila* PPK model (Fig. 6A). Furthermore, we used the resolved ASIC structure to predict structural models for all individual *Drosophila ppk* subunits (Fig. 6B). Close inspection of the structure and the overall protein alignment revealed 10 highly conserved cysteines (>90% conservation), which are likely to form up to five disulfide bonds.

We also found that most family members from group V (Fig. 2) have a long unstructured loop without a matched structural template in the resolved vertebrate model (Fig. 7, with the exception of PPK17). Whether this unstructured loop plays a functional role is unknown. However, *ppk* is expressed in type IV multidendritic neurons, which play a role in thermal and mechanical nociception in fly larvae (Adams *et al.*, 1998; Ainsley *et al.*, 2003; Hwang *et al.*, 2007; Kim *et al.*, 2012; Zhong *et al.*, 2010). The recent publication, which implicates *rpk* in mechanosensotive functions in Class III multidendritic neurons, and our finding that *ppk26* is expressed in Class IV multidendritic neurons in a similar pattern to *ppk* suggest that other members of this cluster might be playing similar roles in mechanotransduction pathways. Further, our data raise the intriguing hypothesis that the large unstructured side loop that is a signature of cluster V may be playing a role in mechanosensory functions, possibly by interacting with extracellular matrix proteins (Arnadottir and Chalfie, 2010; Arnadottir *et al.*, 2011; Brown *et al.*, 2008; Chalfie, 2009; Geffeney *et al.*, 2011; Huber *et al.*, 2006; Zhang *et al.*, 2004).

Concluding remarks

Here we show a comprehensive analysis of an emerging and important family of ion channels in the genetically tractable fruit fly model. As the importance of the DEG/ENaC family continues to rise, studies in *Drosophila* could reveal novel insights into the physiological functions of this enigmatic group of ion channels. Taking advantage of the wealth of genetic and evolutionary data in the *Drosophila* group as well as other insect species, we intend to generate novel testable structure-function hypotheses that would likely shed additional light on the physiological functions of these proteins in species ranging from the worm to humans.

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Figures

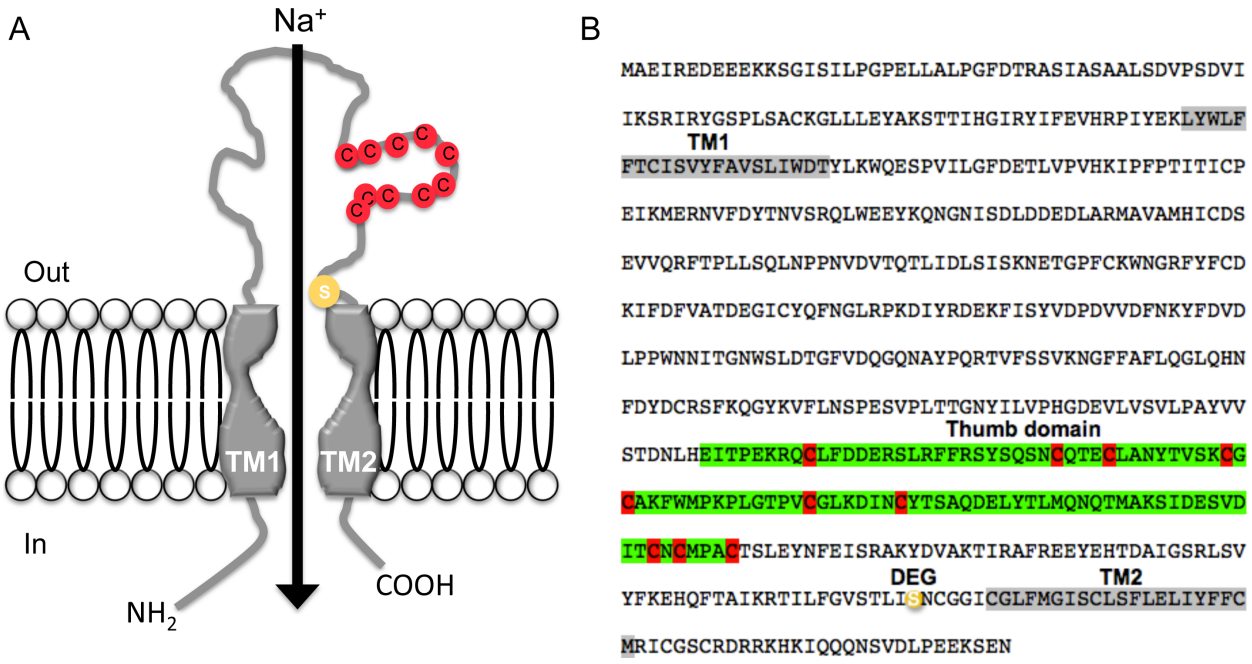

Fig. 1. (*A*) Cartoon depicting a typical DEG/ENaC subunit. TM, transmembrane domain; Red circles represent conserved cysteines; yellow circle represents the "DEG" residue, which in some subunits results in a constitutively open channel state when mutated (Adams *et al.*, 1998; Kellenberger *et al.*, 2002; Snyder *et al.*, 2000; Snyder *et al.*, 1998). (*B*) The protein sequence of PPK, one of the first DEG/ENaC subunits that was identified in the *Drosophila* genome (Adams *et al.*, 1998). Alignment of all the *Drosophila* subunits described in Tables 1 and S1 indicated the presence of a highly conserved cysteine-enriched domain (also see Fig. 7A, thumb domain), highlighted in green. Conserved cysteines are highlighted in red; DEG, a predicted "deg" residue, is highlighted in yellow. TM1 and TM2 represent the predicted transmembrane domains 1 and 2, respectively.

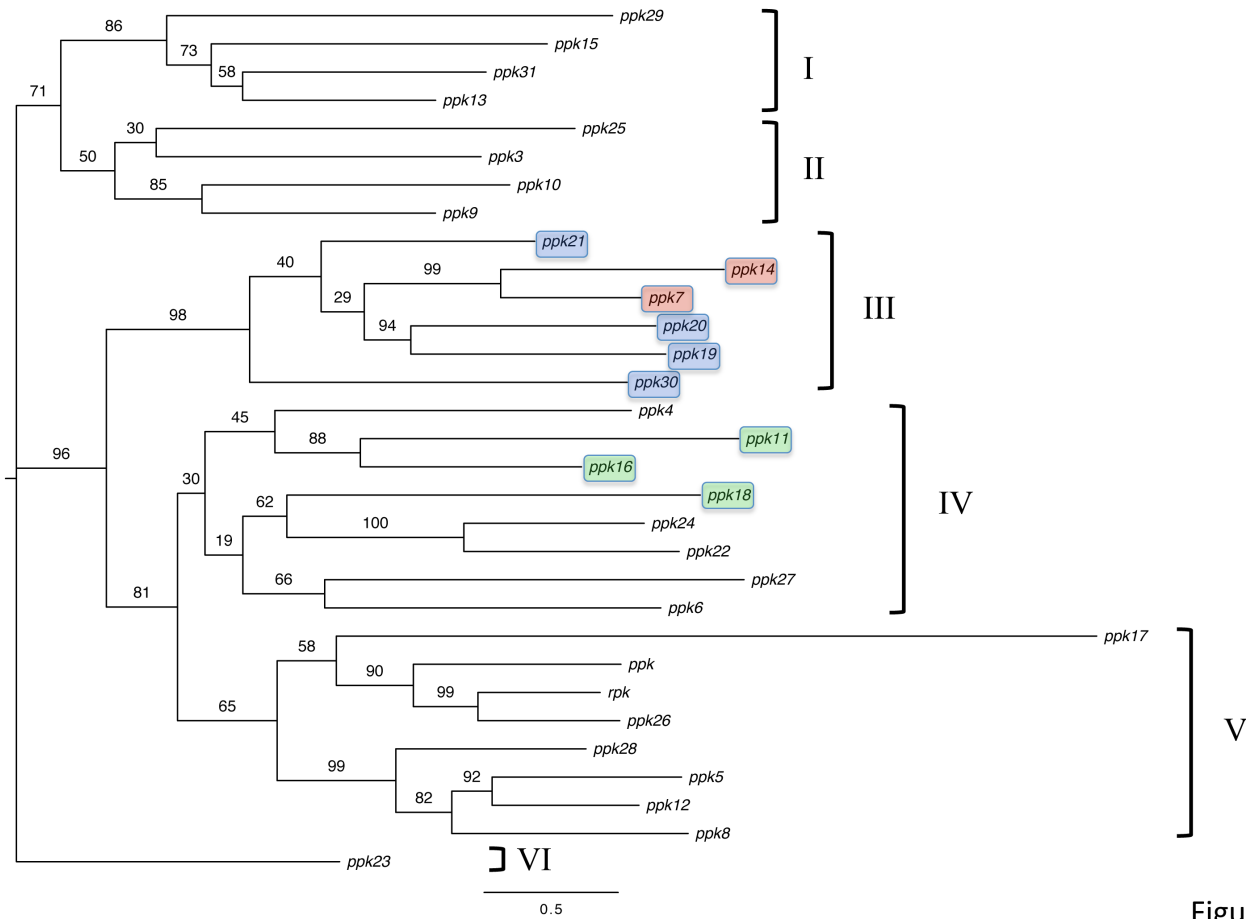

Fig. 2. Maximum-likelihood unrooted phylogenetic tree inferred from multiply aligned amino acid sequences for *D. melanogaster* DEG/ENaC *ppk* genes. 31 DEG/ENaC amino acid sequences are divided into 6 clusters and labeled as groups I-VI. Bootstrap values are given on branches and amino acid substitution rate is given at the bottom of the figure. Colors represent chromosomally clustered subunits (See Fig. 5 for details).

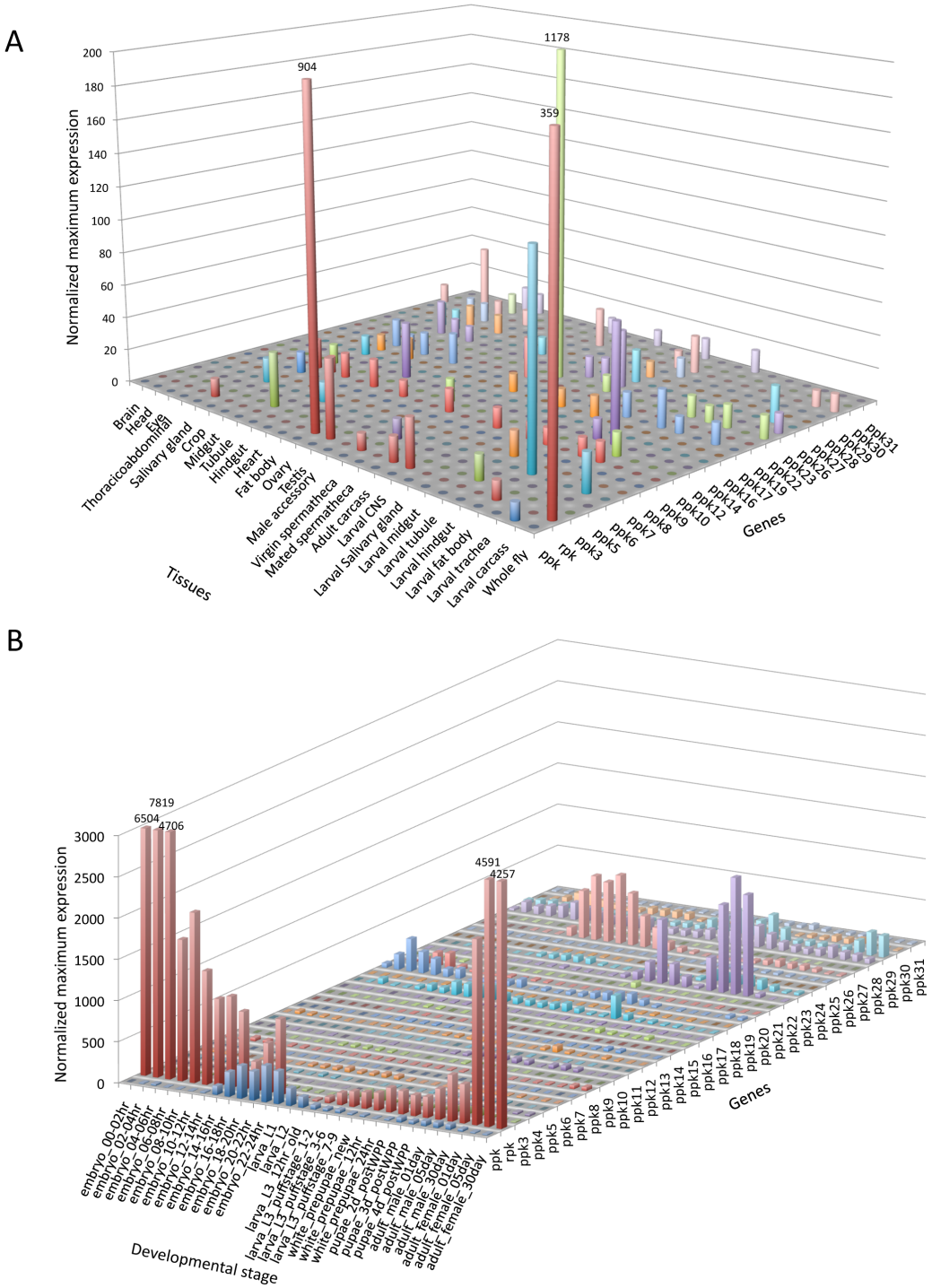

Fig. 3. (*A*) Spatial expression patterns of *ppk* genes. Microarray expression data were extracted from FlyAtlas (Chintapalli *et al.*, 2007). Expression represents the average signal from four independent microarrays. (*B*) Temporal expression patterns of *ppk* genes. Data were extracted from the modENCODE RNA-seq database (Celniker *et al.*, 2009). Expression levels are represented as $\log_2$ values of the original coverage. Numbers at the tops of truncated bars show actual expression values.

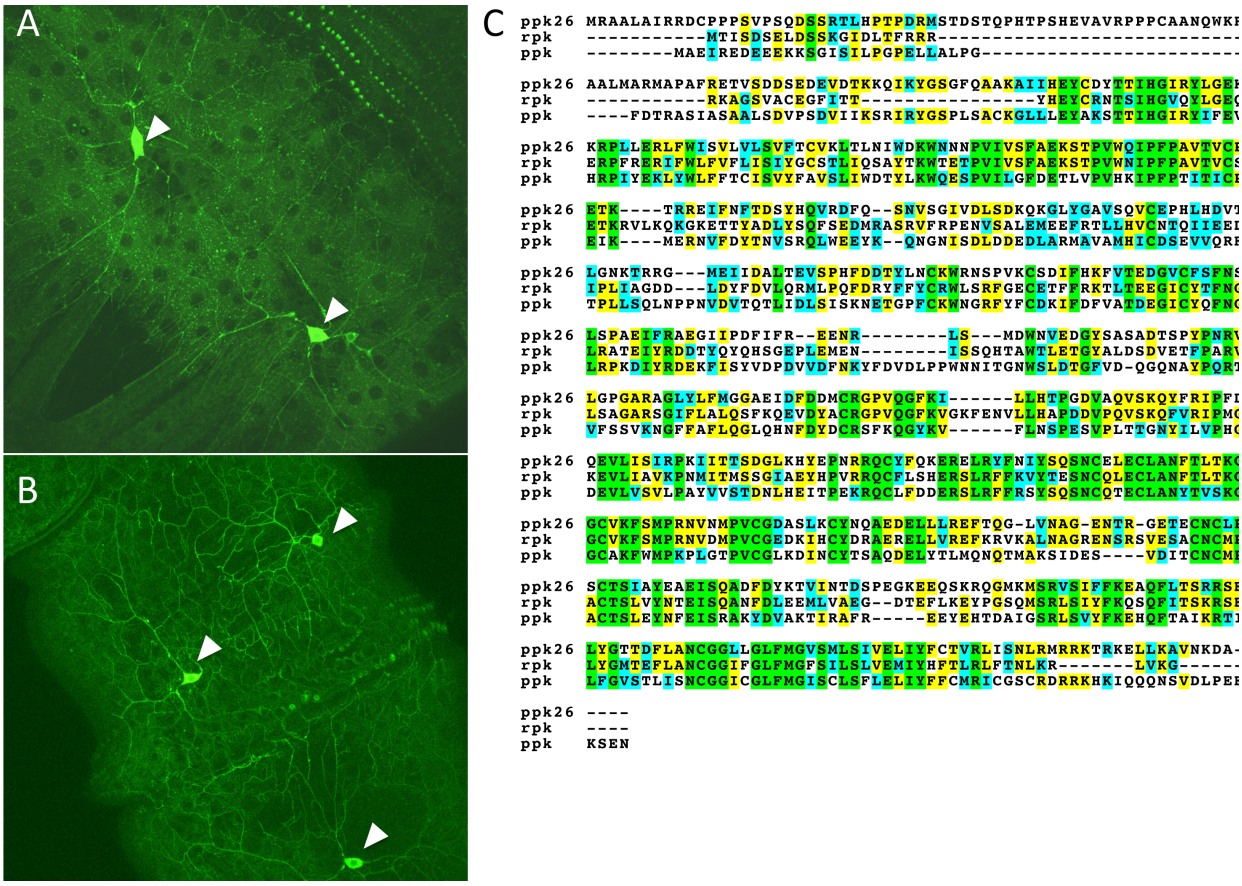

Fig. 4. *ppk* and *ppk26* expression in larval multidendritic neurons. *(A) ppk-GAL4 x UAS-mCD8::GFP. (B) ppk26-GAL4 x UAS-mCD8::GFP*. White arrows indicate cell body. *(C)* Alignment of *ppk*, *rpk*, and *ppk26* amino acid sequence. Green, residues are conserved across all proteins examined; yellow, residues are conserved in some species; blue, conserved substitutions.

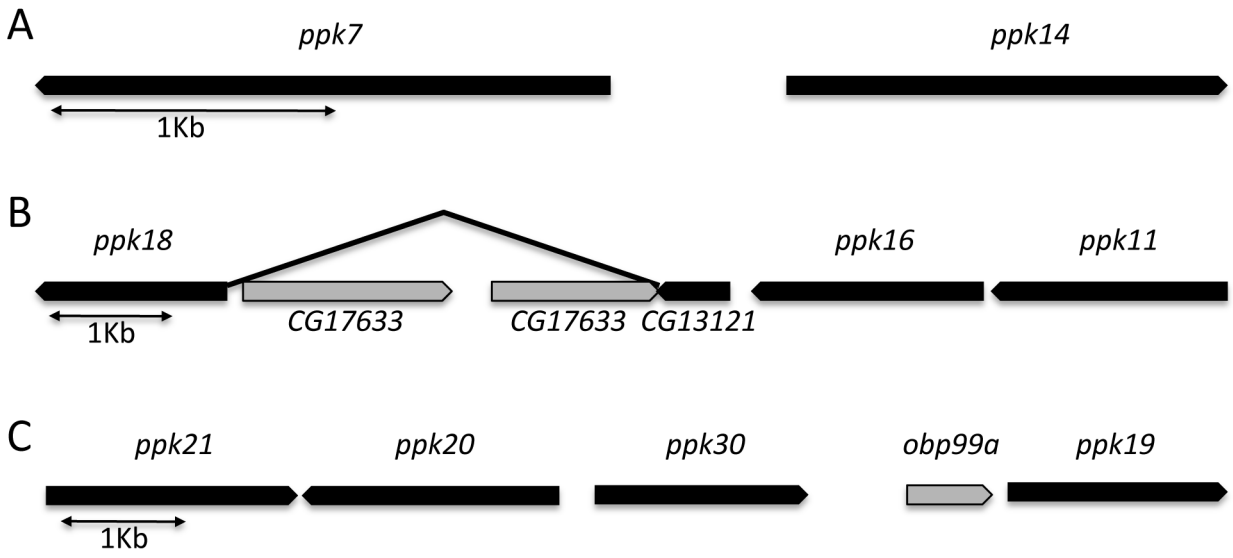

Fig. 5. Chromosomal clusters of *ppk* genes. (*A*) Cluster of *ppk7* and *ppk14* located at 2L: 26C3-26C3. (*B*) Cluster of *ppk18*, *ppk16*, and *ppk11* located at 2L: 30C8-30C9. Note that although CG13121 is currently annotated as a separate gene, molecular analyses of mRNA clones indicate that it is part of the *ppk18* locus (not shown). (*C*) Cluster of *ppk21*, *ppk20*, *ppk30*, and *ppk19* located at 3R: 99B6-99B7. Black boxes, *ppk* genes; gray boxes none-*ppk* genes.

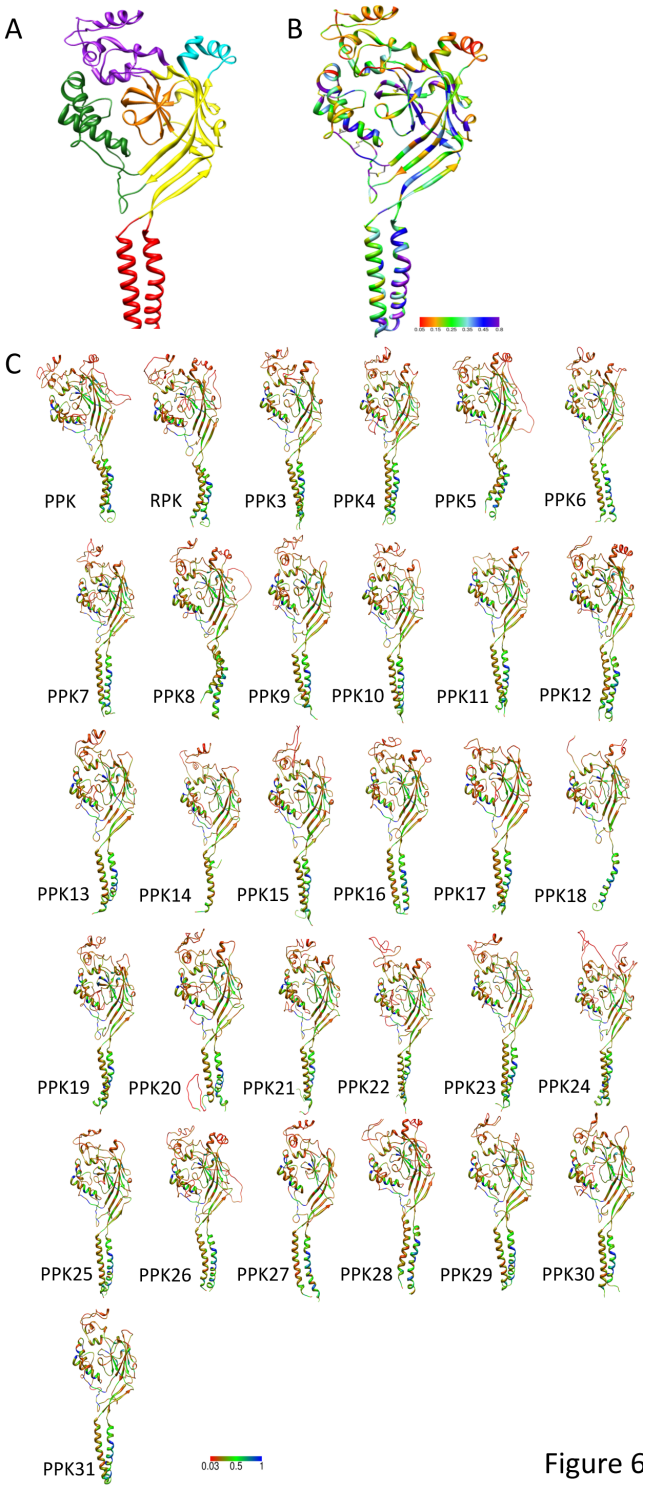

Figure 6

Fig. 6. Structural modeling of the *ppk* family in *Drosophila*. *(A)* Domain organization of the chicken ASIC1a subunit (Jasti *et al.*, 2007) Red: TM1 (left helix), TM2 (right helix); Yellow: Palm; Cyan: Knuckle; Orange: beta-ball; Purple: Finger; Green: Thumb. *(B)* ASIC1a subunit rendered by conservation information from its alignment with the *ppk* family. The regions colored in purple are highly conserved residues, while those colored in red are most variable in the alignment. *(C)* Predicted structure for all *Drosophila* PPK subunits. The rainbow scale represents the residue conservation scores. The regions colored in red are most variable while regions in blue are highly conserved.

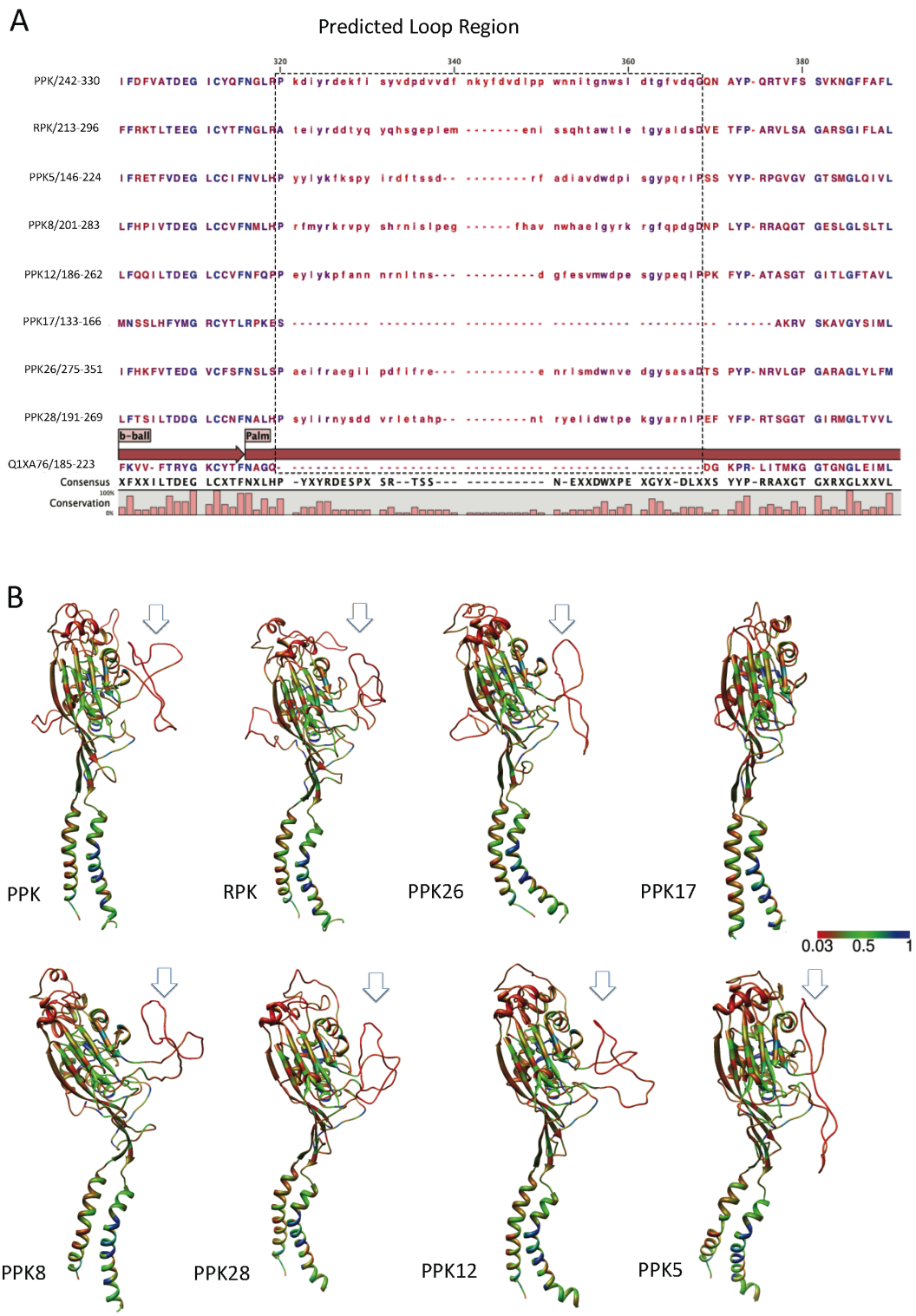

Fig. 7 *(A)* The alignment of individual subunits from *ppk* subfamily Group V (for full Group V alignment see Fig. S1). The dashed frame marks the unstructured loop region. Note that PPK17 does not have the unstructured loop region. Q1XA76 is the chicken ASIC Uniprot Accession ID. Consensus sequence was built from the majority of the aligned residues. The bars in the bottom represent conservation percentage after alignment. *(B)* Unstructured loop region in the subfamily Group V. Predicted structures for all *D. melanogaster* PPK subunits are shown in Fig. 6. The rainbow scale represents residue conservation as in Fig. 6.

Table 1. *ppk* genes identified in the *Drosophila melanogaster* genome.

Name	Symbol	Alternative name	CG#	FB ID	Location
pickpocket 1	*ppk*	*ppk1*	*CG3478*	FBgn0020258	2L: 35B1-35B1
ripped pocket	*rpk*	*ppk2*	*CG1058*	FBgn0022981	3R: 82C5-82C5
pickpocket 3	*ppk3*		*CG30181*	FBgn0050181	2R: 59E3-59E3
Nach	*Nach*	*ppk4*	*CG8178*	FBgn0024319	2R: 53C14-53C14
pickpocket 5	*ppk5*		*CG33289*	FBgn0053289	3L: 78D5-78D5
pickpocket 6	*ppk6*		*CG11209*	FBgn0034489	2R: 56F11-56F11
pickpocket 7	*ppk7*		*CG9499*	FBgn0031802	2L: 26C3-26C3
pickpocket 8	*ppk8*		*CG32792*	FBgn0052792	X: 3D6-3D6
pickpocket 9	*ppk9*		*CG34369*	FBgn0085398	2R: 58A4-58A4
pickpocket 10	*ppk10*		*CG34042*	FBgn0065110	2L: 31E3-31E4
pickpocket 11	*ppk11*		*CG34058*	FBgn0065109	2L: 30C8-30C9
pickpocket 12	*ppk12*		*CG10972*	FBgn0034730	2R: 58E1-58E1
pickpocket 13	*ppk13*		*CG33508*	FBgn0053508	2L: 39A1-39A1
pickpocket 14	*ppk14*		*CG9501*	FBgn0031803	2L: 26C3-26C3
pickpocket 15	*ppk15*		*CG14239*	FBgn0039424	3R: 97B1-97B1
pickpocket 16	*ppk16*		*CG34059*	FBgn0065108	2L: 30C8-30C8
pickpocket 17	*ppk17*		*CG13278*	FBgn0032602	2L: 36A14-36A14
pickpocket 18	*ppk18*		*CG13120*	FBgn0032142	2L: 30C7-30C8
pickpocket 19	*ppk19*		*CG18287*	FBgn0039679	3R: 99B7-99B7
pickpocket 20	*ppk20*		*CG7577*	FBgn0039676	3R: 99B7-99B7
pickpocket 21	*ppk21*		*CG12048*	FBgn0039675	3R: 99B6-99B6
pickpocket 22	*ppk22*		*CG31105*	FBgn0051105	3R: 96B1-96B1
pickpocket 23	*ppk23*		*CG8527*	FBgn0030844	X: 16B4-16B4
pickpocket 24	*ppk24*		*CG15555*	FBgn0039839	3R: 100B9-100B9
pickpocket 25	*ppk25*	*lounge lizard (llz)*	*CG33349*	FBgn0053349	2R: 42E1-42E1
pickpocket 26	*ppk26*		*CG8546*	FBgn0035785	3L: 66A1-66A1
pickpocket 27	*ppk27*		*CG10858*	FBgn0035458	3L: 63E9-63E9
pickpocket 28	*ppk28*		*CG4805*	FBgn0030795	X: 15A9-15A10
pickpocket 29	*ppk29*		*CG13568*	FBgn0034965	2R: 60B6-60B6
pickpocket 30	*ppk30*		*CG18110*	FBgn0039677	3R: 99B7-99B7
pickpocket 31	*ppk31*		*CG31065*	FBgn0051065	3R: 97E5-97E6

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